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THE UNIVERSITY OF ALBERTA

SYNTHESIS AND PHYSICAL STUDIES OF TRIDENTATE MODELS FOR CARBONIC ANHYDRASE

by

ⓒ JOAN HUGUET

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

IN CHEMISTRY

EDMONTON, ALBERTA
FALL, 1980

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

SYNTHESIS AND PHYSICAL STUDIES
OF TRIDENTATE MODELS FOR CARBONIC ANHYDRASE

submitted by JOAN HUGUET in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

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Dedico aquesta tesis als meus
pares com a mostra d'agraïment per haber-me
donat l'oportunitat d'estudiar

ABSTRACT

Carbonic Anhydrase (C.A.) is a zinc-containing enzyme, widely distributed in nature, whose physiological role is to accelerate the reversible hydration of carbon dioxide ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$). C.A. also catalyzes other reactions such as hydration of aldehydes and hydrolysis of esters. According to X-ray diffraction studies, its active site contains a zinc ion coordinated, at the base of a deep cleft in the protein, to three histidine imidazole residues and a water molecule or hydroxide ion. Several mechanisms of action have been proposed for C.A., and some models studied to verify the likelihood of some of these mechanisms. None of these models, however closely resembles the active site of the enzyme.

In this work several tridentate ligands [9-17, 29(a-c)] containing imidazole moieties have been synthesized and their physicochemical properties studied and compared to those of the enzyme. First of all, the models must be shown to bind metals: therefore their metal binding ability has been studied using a potentiometric method. The metal binding constants found are consistently 3-4 pK units lower than those of apo-carbonic anhydrase. Second, the mode of the metal binding has to be tridentate. In order to check this, nmr experiments of ligand solutions with varying Zn(II)

concentrations have been performed. The small ligands 9-12 exist as 2:1 or 1:1 metal complexes, the latter being favored at high metal concentrations. Deleterious 2:1 ($L:M^{++}:L$) binding can be overcome by placing large alkyl groups at the 4,5-imidazole positions. However such substitution in the *tris*-imidazole carbinol series appears to lead to facile dehydration to produce highly colored fulvene-like materials. Such dehydration can be overcome by removing the carbinol OH group or methylating the imidazole nitrogen. However, the former substitution of OH by H produces an extremely easily air-oxidized methane, and the latter N-methylation leads to rather poor metal binding ability. Ligands 17 and 29(a-c) avoid these problems by inserting a methylene unit between the carbinol group and the imidazole ring (17) or by substituting the carbinol group by a phosphorous (phosphines 29). Phosphine 29c shows from the nmr study the formation of a symmetric, tridentate, 1:1 complex with Zn^{++}.

Thirdly, since the coordination number around the metal should be four to approximate the pseudotetrahedral arrangement in the active site of C.A., the UV-visible spectra of the ligands using Co(II) as a probe have been recorded. Only carbinol 17 and phosphine 29c show the ability to form four and/or five coordinate complexes; the others appear to form octahedral complexes predominantly. Fourthly, since the activity of the enzyme has been associated with the basic form with pK_a ~ 7, the zinc complexes of these ligands have been titrated with base to determine whether they have a titratable group with such a pK_a.

Finally, the more promising ligands have been checked for catalytic activity using reactions which are known to be catalyzed by the enzyme, such as PNPA hydrolysis and CH_3CHO and CO_2 hydration. Ligands 17 and 29c show some catalytic activity towards CO_2 hydration. Although encouraging, this catalytic activity is small compared to the enzyme, suggesting that the creation of a simple metal binding cavity might not be enough to account for full enzymatic activity, and that some other factors such as hydrogen bonding in the vicinity of the metal might have to be considered in the design of new ligand models.

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I want to thank Prof. R.S. Brown for being a good friend in addition to being my research supervisor. Also for his guidance during my graduate career and invaluable assistance in the preparation of this thesis.

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Special thanks to Ms. Diane Dowhaniuk for her beautiful job in typing this manuscript.

Magda, aquesta tesis l'hauriam de firmar els dos, perque encara que sembli que tot el treball l'he fet jo, tu ja saps que l'hem fet a mitges. Gràcies per estar tant aprop meu durant aquests quatre anys i per aguantar sense protestar els rollos sobre el zinc i les enzimes, i totes les altres xaladures de químic.

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LIST OF ABREVIATIONS

C.A.	Carbonic Anhydrase
HCAC	Human Carbonic Anhydrase Isozyme C
HCAB	Human Carbonic Anhydrase Isozyme B
BOVCA	Bovine Carbonic Anhydrase
Tris	*Tris*(hydroxymethyl)methylamine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PNPA	*p*-nitrophenyl acetate
DMF	N,N-dimethylformamide
NMR	Nuclear Magnetic Resonance

I. INTRODUCTION

A. CARBONIC ANHYDRASE

Carbonic Anhydrase[1] was first isolated from blood in 1932 by Meldrum and Roughton[2] and found to catalyze the reversible hydration of carbon dioxide. Although the enzyme is present in most organisms, and is found in many different tissues of plants and animals, most of the present knowledge about its molecular properties and mechanism is based on studies of the forms isolable from human and bovine erythrocytes[1e], the enzyme being the major protein component of the red blood cell other than hemoglobin[1f]. It is involved in a variety of physiological functions where its specific catalytic role is to facilitate the interconversion of carbon dioxide and bicarbonate[3].

The human erythrocyte enzyme is comprised of three distinct isozymes designated A, B, and C in relative abundance 5, 83, and 12%, respectively.

All three variants consist of about 260 aminoacids in a single polypeptide chain, contain one zinc ion per molecule, and have molecular weights near 30000. Forms A and B are indistinguishable in terms of their specific activities towards the hydration of CO_2 and aminoacid compositions[1e]. However, there is only a 59% homology between the amino acid sequences of human carbonic anhydrases B and C, and the human C isozyme has a maximal turnover number with respect to the hydration of CO_2 which is five times as large as that of the human B[1e]. Bovine erythrocyte carbonic anhydrase consists of two isozymes A and B in relative abundance 20 and 80% respectively, that appear to have identical aminoacid compositions and are equally highly active towards common substrates[1e]. It has been suggested[3] that the low activity form arises from a gene duplication that took place early in mammalian evolution, perhaps 100-150 million years ago.

1. X-RAY STRUCTURE

The complete structures of the human isozymes B and C have been deduced from high-resolution X-ray studies on enzyme crystals obtained from 50 mM. Tris-sulfate buffer solutions, pH 8.5[4,5]. The tertiary structures of the two isozymes are very similar. The enzymes are ellipsoids of dimensions 41 x 42 x 55 Å, and the zinc ion is near the center of the molecule at the bottom of a 12 Å deep conical cavity, where it is bound to the protein through the nitrogen atoms of three histidine imidazole ligands (His 94, 96, and 119). Of these three ligands, histidine 94 differs from the other two in that it is 0.4 Å further from the zinc ion (TABLE I).

TABLE I

Zinc ligands in HCAC[6]

Ligand	Zinc-ligand distance (Å)
His 94 (3' N)	2.4
His 96 (3' N)	2.0
His 119 (1' N)	2.0
H_2O/HO^-	1.9

Either a water molecule or a hydroxide ion occupies a fourth position around the zinc, thus completing a somewhat distorted tetrahedral coordination in which the greatest deviation from the regular tetrahedral angles is about 20°. The water molecule directly ligated to the metal ion is hydrogen bonded to threonine 199, which in turn is hydrogen bonded to the buried glutamic acid 106. The fact that Thr 199 forms hydrogen bonds with most inhibitors suggests that it plays an active role in catalysis. Apart from the histidine ligands, other residues in the active site that are common to both isozymes B and C[5b] are Thr 199, Pro 201, Pro 202, His 64, Gln 92 and those involved in a hydrogen-bonded sequence: His 119 - Glu 117 - His 107 - Tyr 194 - Ser 29 - Trp 209 (Figs. 1 and 2).

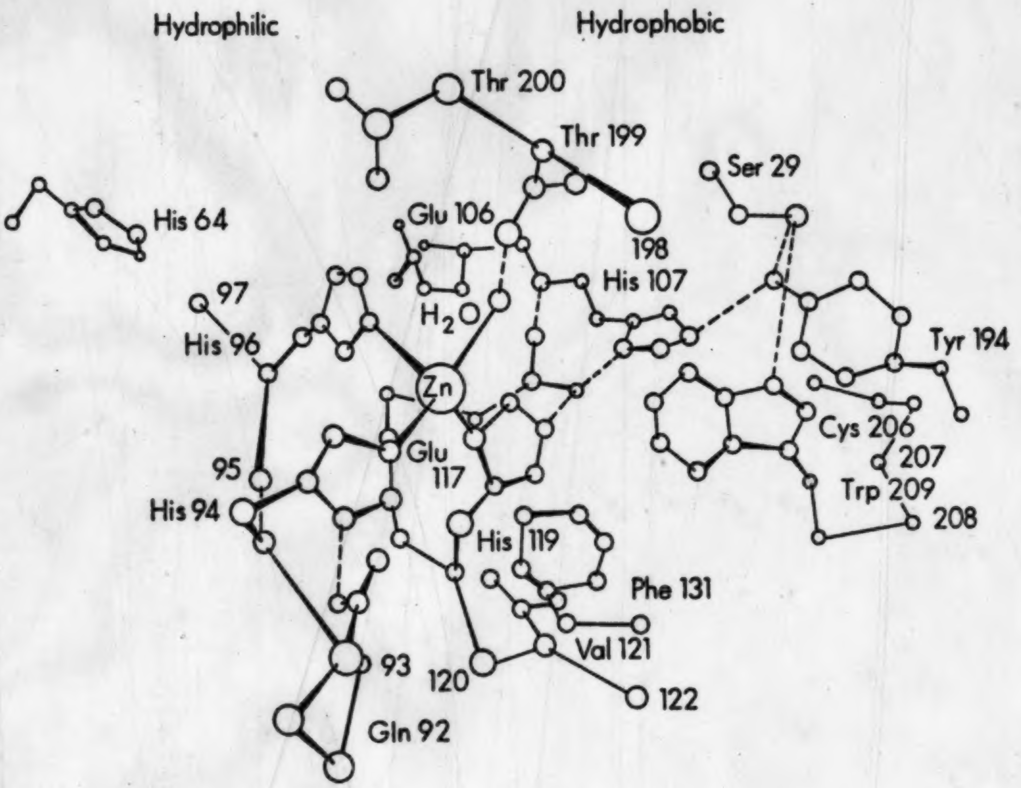
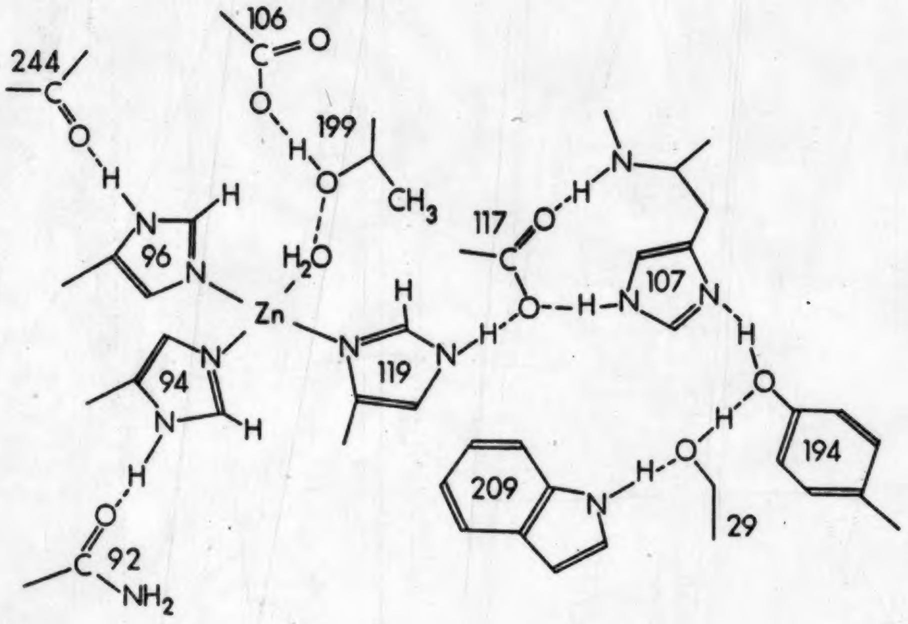

Hydrophilic
Hydrophobic
Thr 200
Thr 199
Ser 29
His 64
Glu 106
198
His 107
97
H_2O
His 96
Zn
Tyr 194
Cys 206
207
Glu
117
95
Trp 209
208
His 94
His
119
Phe 131
Val 121
93
120
122
Gln 92

Fig. 1. Part of the active site of human carbonic anhydrase C; redrawn from ref. 1f.



244
106
199
CH$_3$
H$_2$O
Zn
96
94
119
117
107
194
209
29
92
NH$_2$

Fig. 2. A schematic view of part of the active site of carbonic anhydrase, showing the three imidazole zinc ligands and the hydrogen bonding network around the active site. These features are common to human carbonic anhydrases B and C[5c]; redrawn from ref. 23.

2. METAL ION SPECIFICITY

The zinc ion in carbonic anhydrase can be removed at low pH. While the apoenzyme undergoes no gross structural changes relative to the holoenzyme, it is catalytically inactive. The apoenzyme can be reconstituted with other divalent metal ions and these occupy the zinc site; significant restoration of catalytic activity is brought about only by Zn(II) and Co(II)[1a,1c,4c,7].

3. Co(II) CARBONIC ANHYDRASE (Co(II) C.A.)

The Co(II) C.A. has a reddish blue color with a maximal molar absorptivity of 300-400 M^{-1} cm^{-1}[8a,b,c]. Its absorption spectrum displays a band structure however, which does not correspond to that of any model Co(II) complexes. At pH 9 there are four widely split maxima at 520, 555, 615, and 645 nm indicative of a distorted coordination geometry around the metal, corresponding to a four and/or five coordinate ligand field[1a,1b,1e,9,7b,10]. There appears to be a change in geometry surrounding the Co(II) ion as the pH is lowered from 9 to 6: the widely split spectrum of the Co(II) enzyme is present only under alkaline conditions.

The change in absorptivity at 640 nm has been observed[8a,8b,8c] to follow a titration curve with a single pK_a around 7 when measurements are performed in buffered solutions or in the presence of salts to keep the ionic strength constant. The catalytic activity of both the native and cobalt substituted enzymes also depends on pH[8d,8e,12b]. The pH dependences of both the spectra and the catalytic activity have always been attributed to the same ionizing group[1a,1c,1e].

However, it has been shown recently[11] that in the absence of buffers and monovalent anions the pH dependence of the electronic spectra of Co(II) substituted C.A. is not as simple as expected for a single dissociating group. It has been suggested[11] therefore, that there is no reason to ascribe all the pH dependent properties of the enzyme to only one dissociating group: one group could be responsible for some properties and some other for other properties, in such a way that a more complex explanation should be sought.

4. CATALYTIC PROPERTIES OF CARBONIC ANHYDRASE

The only known physiological function for C.A. is the catalysis of the interconversion of carbon dioxide and bicarbonate[1e]. In vitro, the enzyme catalyzes a range of reactions in which nucleophilic attack of oxygen at an electrophilic center occurs[1g]. These include the hydration of carbon dioxide[12], aldehydes[13], pyruvic acid[14] and alkyl pyruvate esters[15], the hydrolysis of some carboxylic, carbonic, sulfonic and phosphoric esters[16], and the hydrolysis of carbobenzoxy and sulfonyl chlorides[16c,17], of 1-fluoro-2,4-dinitrobenzene[18] and of phenyl N-methylacetimidate[19].

The enzyme-catalyzed reactions can be formally analyzed in terms of the elementary classical Michaelis-Menten scheme, according to which the velocity of enzymatic catalysis, v_{enz} is given by $k_{cat}[E]_o/(1+K_M/[S])$, where k_{cat} is the turnover number, $[E]_o$ is the initial concentration of enzyme, K_M is the Michaelis constant, and [S] is the concentration of substrate.

Some selected values of the Michaelis parameters for CO_2 and acetaldehyde hydration, and PNPA hydrolysis are given in TABLE II.

BOVCA and HCAC are kinetically similar in some respects and have sigmoidal pH vs. k_{cat} profiles for CO_2 hydration over the pH range between 6 and 9, consistent with the titration of a group with pK_a around neutrality. The situation is a little more complex for HCAB, where the state of ionization of additional groups appears to influence the rate.

TABLE II

Michaelis parameters for the C.A.-catalyzed hydration of CO_2 and CH_3CHO and hydrolysis of PNPA[1e,1g].

Substrate	Isozyme	pH	k_{cat} (sec^{-1})	K_M (mM)
CO_2	HCAC	7.05	6.2×10^5	14
	HCAB	7.05	1.5×10^4	2.6
	BOVCA	6.75	4.2×10^5	15.5
CH_3CHO	BOVCA	7.20	800	650
PNPA	HCAC	8.1	20	8.7
	HCAB	8.2	2.5	4.5
	BOVCA	7.0	.13	1.4
	BOVCA	9.0	1.1	8.6

B. PROPOSED MECHANISMS FOR CARBONIC ANHYDRASE

A variety of detailed chemical mechanisms for the C.A.-catalyzed hydration of CO_2 has been suggested which appear to be compatible with most, if not all of the available physicochemical information[1].

The activity of C.A. around neutral pH is governed by the ionization of at least one group with pK_a near 7. The enzyme-catalyzed CO_2-HCO_3^- interconversion at around neutral pH can be schematized as in equation 1.

$$E + CO_2 \xrightleftharpoons{H_2O} EH^+ + HCO_3^- \qquad (1)$$

This protonated enzyme (EH^+) must undergo ionization to regenerate the active form of the enzyme (E) before the turnover cycle is complete (equation 2).

$$EH^+ \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E + H^+ \qquad (2)$$

This transformation must occur at a rate which is greater than, or equal to, the rate of turnover. ($k_{cat} \approx 5 \times 10^5 \text{ sec}^{-1}$, TABLE II). Then k_f must be $\geq 5 \times 10^5 \text{ sec}^{-1}$. Since the interconversion of the acidic and basic forms of the enzyme involves an ionization of apparent $pK_a \sim 7$, then

$$k_r = k_f/K_E = 5 \times 10^5 \text{ sec}^{-1}/10^{-7} \text{ M} = 5 \times 10^{12} \text{ M}^{-1} \text{ sec}^{-1} \qquad (3)$$

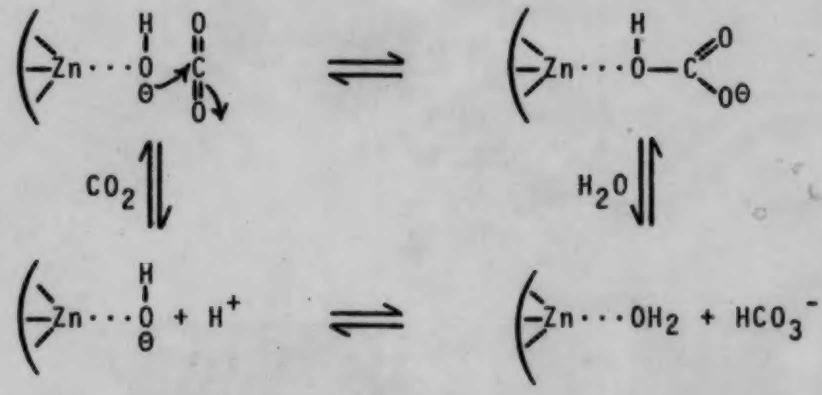
This value for k_r exceeds the diffusion-limited combination rates observed for small molecule acid-base neutralization reactions in aqueous solutions[21] by a factor of 100. In order to explain this paradox, protein- and/or buffer-mediated proton transfer needs to be invoked[22].

Four distinct alternatives have been suggested[23] for the ionizable group. The proposed mechanisms are divided into categories according to this group: (1) a metal-coordinated water molecule ionizing to OH^- (mechanisms W1, W2, W3, W4, and W5); (2) a titratable imidazole group associated with the metal ion via one or more water molecules (mechanisms IH1, IH2); (3) a neutral imidazole group ionizing to a metal-coordinated imidazolate anion (mechanism I1); (4) the partially buried carboxyl group from Glu 106, connected to a metal-coordinated water molecule via a hydrogen bond system: Glu 106 - Thr 199 - $H_2O \cdot Zn$[20] (mechanism G1).

[For a complete discussion on the validity of each one of these mechanisms see refs. 1d, 1e, 1f, and 1h. The charge in the metal ion has been omitted for simplicity.]

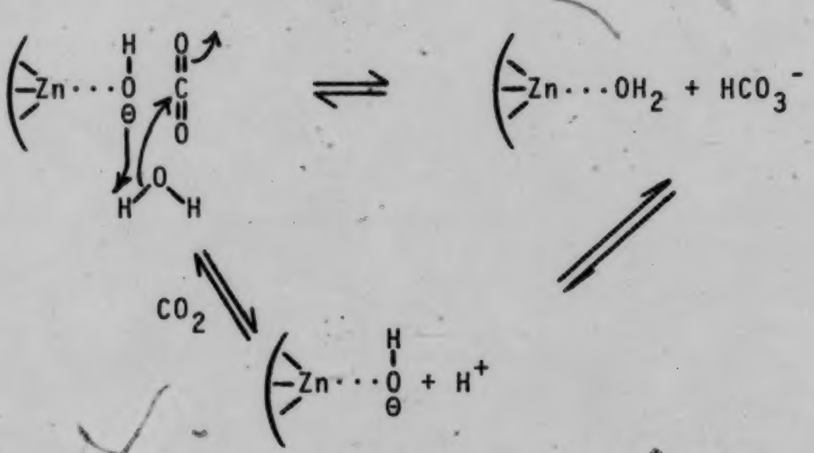
SCHEME I

Mechanism W1. Nucleophilic attack by Zn-OH[1a,1j,24]



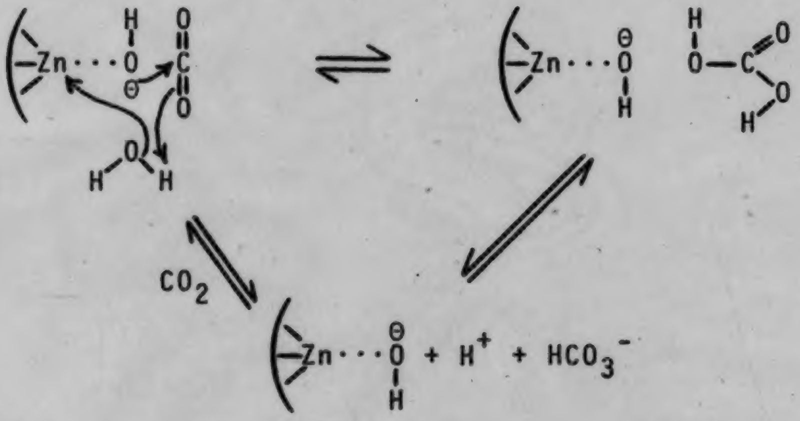
SCHEME II

Mechanism W2. General base attack by H_2O assisted by Zn-OH[25]


Zn
H
O
C
O
O
H
O
H
$\rightleftharpoons$
Zn···OH$_2$ + HCO$_3^-$
CO$_2$
Zn···O + H$^+$

SCHEME III

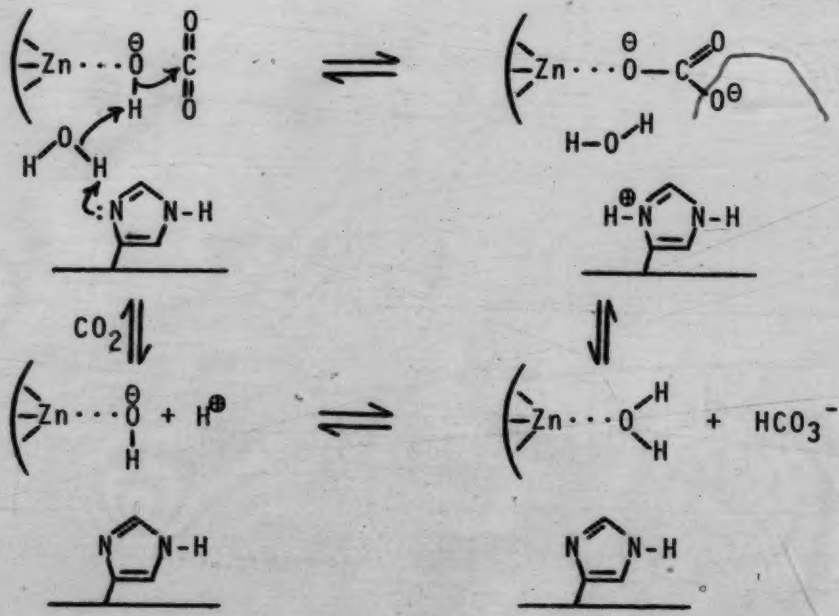
Mechanism W3. Nucleophilic attack by Zn-OH with concomitant proton transfer[26]


Zn
H
O
C
O
O
H
O
H
$\rightleftharpoons$
Zn···O
H
O—C
O
O
H
CO$_2$
Zn···O + H$^+$ + HCO$_3^-$

In mechanisms W1, W2, and W3 CO_2 never binds to Zn and the histidine ligands play the passive role of anchoring the metal. The crucial role of Zn in these mechanisms is to acidify its ligated water molecule. This water molecule is converted into a zinc-bound hydroxide ion, which is nucleophilic enough to attack a CO_2 molecule either directly (W1, W3) or through a general base mechanism (W2). Mechanism W3 was originally proposed in an attempt to avoid any potential difficulties associated with proton transfer between the enzyme and solvent during the catalytic cycle. It can be demonstrated[12] that this mechanism is incompatible with the high turnover number, unless the pK_a of carbonic acid in the active site is increased by 2 units.

SCHEME IV

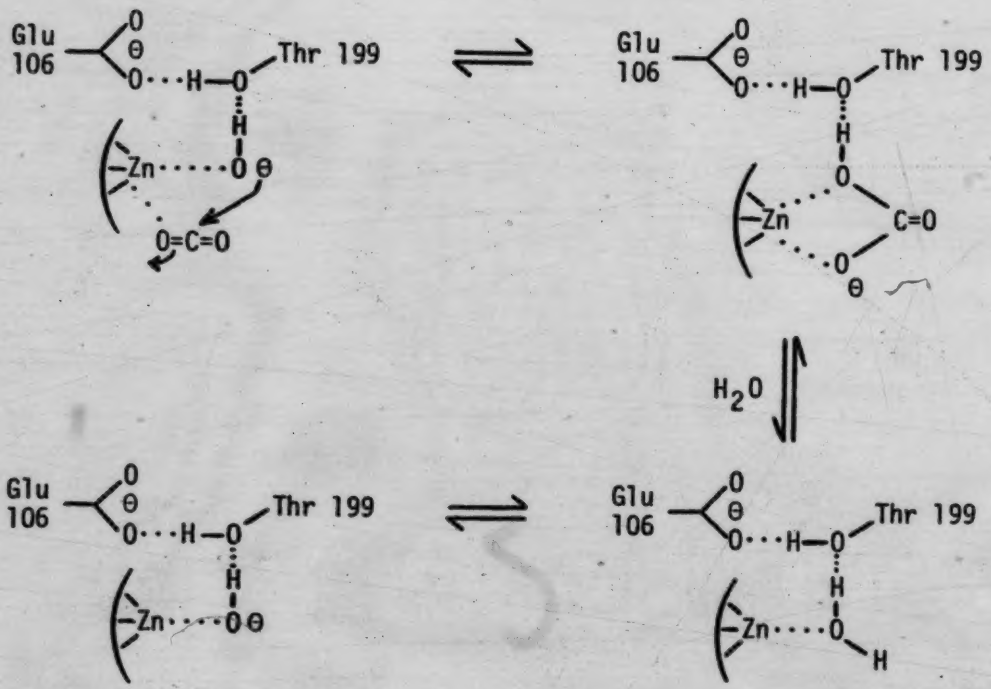
Mechanism W4. General base attack by Zn-OH assisted by His 64[27]



The Zn-OH species is $\sim 10^8$ times less basic than free OH^-. It is reasonable to expect that it is somewhat less nucleophilic too. Mechanism W4 was proposed to overcome this difficulty: the incipient zinc oxide would be a better nucleophile than the zinc-hydroxo complex.

SCHEME V

Mechanism W5. Nucleophilic attack by Zn-OH assisted by hydrogen bonding with Thr 199 and Glu 106[20].

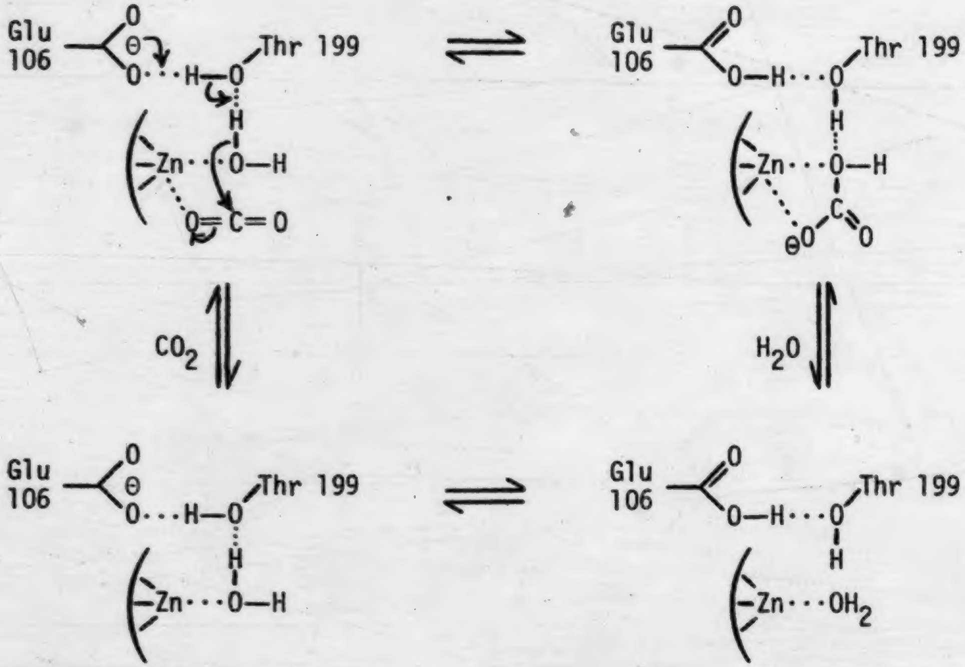

Glu 106
Thr 199
Zn
O=C=O
Glu 106
Thr 199
Zn
C=O
H₂O
Glu 106
Thr 199
Zn
Glu 106
Thr 199
Zn

This is really a more elaborate W1 mechanism. The distinguishing features of this one are twofold. First, activation of the nucleophile through hydrogen bonding with Thr 199 and Glu 106. The x-ray structures of the native enzyme and the enzyme-inhibitor complexes[4,5] indicate that Thr 199 plays a role in stabilizing coordination of ligands to the fourth ligand site through hydrogen bonding between the Thr 199 hydroxyl group and the ligand. It is suggested here that Thr 199 plays a similar role during the catalytic transformation[20].

Second, interaction in a five coordinate species between the Zn and an oxygen atom of CO_2, polarizes the O=C=O group $(\mathrm{Zn}\leftarrow\overset{\delta-}{\mathrm{O}}=\overset{\delta+}{\mathrm{C}}=\mathrm{O})$ and activates it to nucleophilic attack. This five-coordination has precedent in the HCAB- imidazole complex[20]. Imidazole, the only known competitive inhibitor for the hydration of CO_2 by C.A., appears to occupy a distant fifth coordination site on the Zn ion, its nearest nitrogen atom being 2.7 Å from the Zn. It sits in a hydrophobic pocket which is believed to be occupied by CO_2 in the catalytic reaction[27b].

SCHEME VI

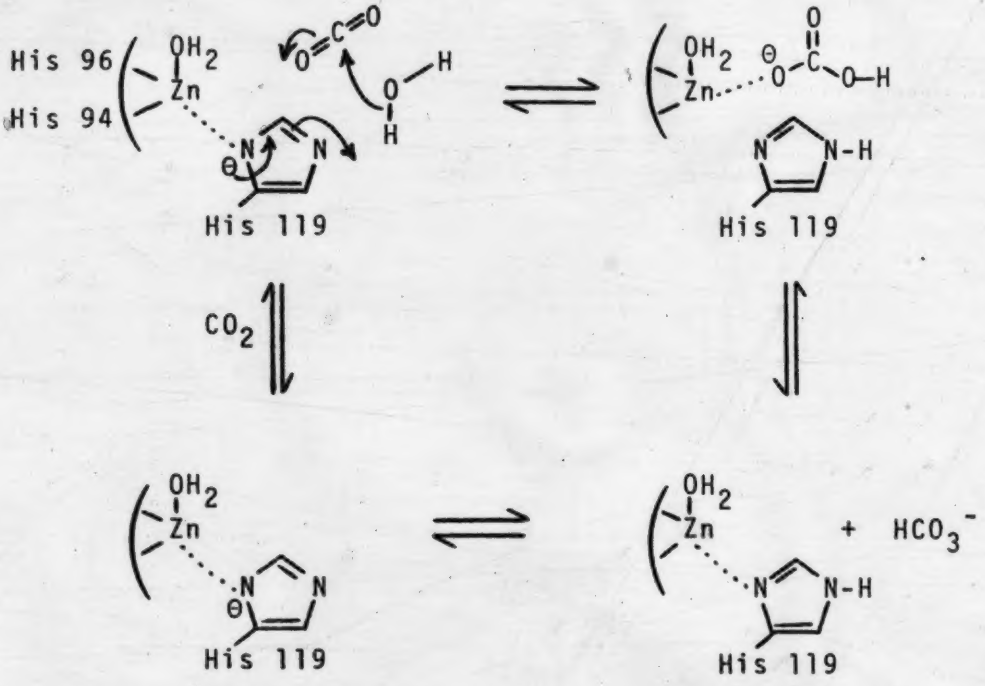
Mechanism G1. General base attack by Zn-OH_2 assisted by Glu 106 through Thr 199[20].



Although very similar to W5 with the difference that here Glu 106 acts as the titrable catalytic group this mechanism suffers from the problem that an exceptionally high pK_a must be assigned to Glu 106 despite its close contact with the positively charged metal ion.

SCHEME VII

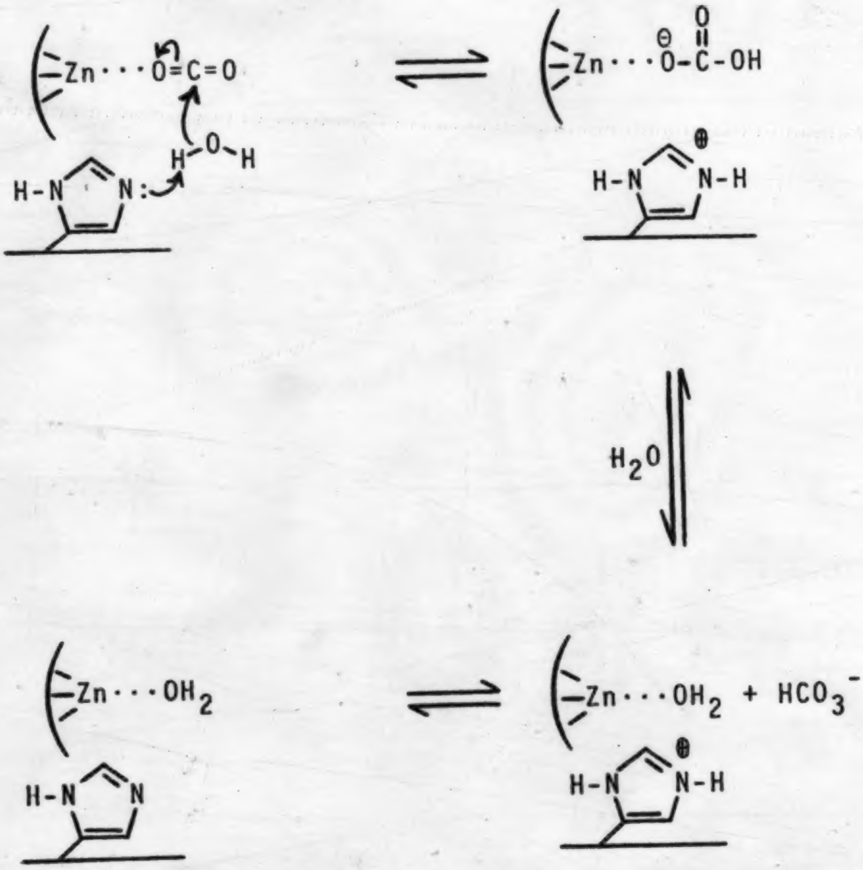
Mechanism II. General base attack by H_2O assisted by His 119 imidazolate anion moiety[28].


His 96
His 94
OH_2
Zn
N
N
His 119
O=C=O
O
H
H
OH_2
Zn
O
C
O
O-H
N
N-H
His 119
CO_2
OH_2
Zn
N
N
His 119
OH_2
Zn
N
N-H
+ HCO_3^-
His 119

This mechanism was originally based on the downfield shift of the C-2 proton of His 94 as pH was increased, following a titration curve with an associated $pK_a \sim 7$. Catalysis is then effected by a strong general base juxtaposed to a powerful Lewis acid.

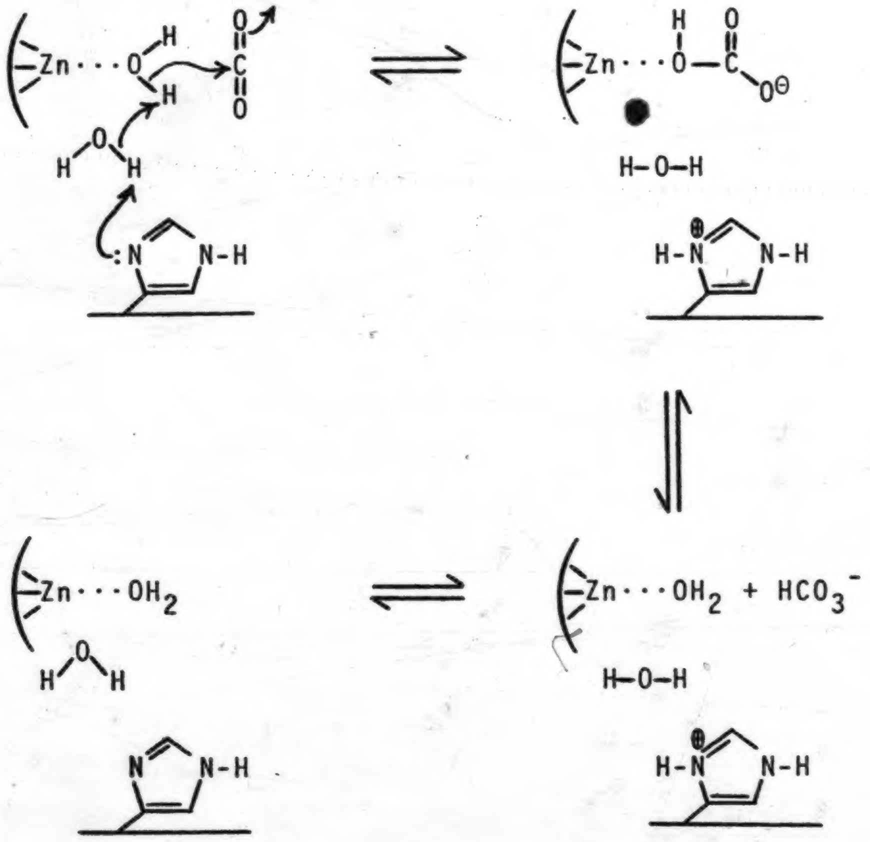
SCHEME VIII

Mechanism IH1. General base attack by H_2O assisted by His 64 imidazole moiety[1d].


Zn···O=C=O
H-O-H
H-N
N:
Zn···O-C-OH
O
H-N
N-H
H_2O
Zn···OH_2
H-N
N
Zn···OH_2 + HCO_3^-
H-N
N-H

SCHEME IX

Mechanism IH2. General base attack by Zn-OH_2 assisted by His 64 imidazole moiety[29].



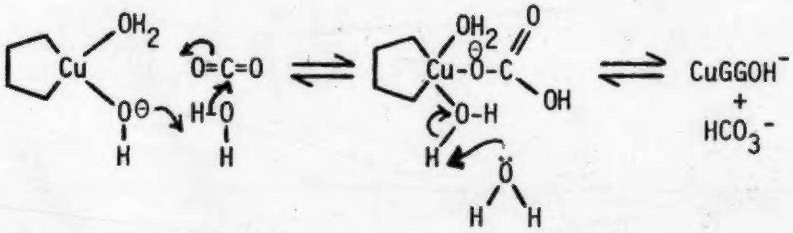
Mechanisms IH1 and IH2 do not require any "exceptional" pK_a for the titrable group since the imidazolium moiety of a histidine has a pK_a of around 7. The main argument against these mechanisms is that alkylation of His 64 with bromopyruvate[30] only brings about a 70% decrease in the activity of HCA. This indicates that the modified imidazole ring must be able to rotate freely for the non-alkylated nitrogen to be positioned appropriately, or that the carboxylate group of the modifying moiety assumes the role of general base if the active site histidine residue alone governs the catalytic behavior around neutral pH.

C. PREVIOUS MODELS FOR CARBONIC ANHYDRASE

According to Woolley[31] a "model is understood as a simple system with a property or properties relating it to the biological system under consideration. Ideally a range of model compounds, structurally controlled to reproduce in varying degrees the property in question, provides a quantitative scale in which the biological system might fall; if it continues to deviate from this scale then it has still not been fully understood. A model system may alternatively be designed to reproduce one of the biological properties of the system, for instance to test the hypothesis that a sufficient condition for the action of an enzyme is a particular conjunction of reacting atoms or groups; if successful this does not confirm that the biological system acts in the same way as the model, but it does show that the action of the simple system is in principle available for nature to make use of in the complex one."

Several models have been studied in order to test for some of the proposed mechanisms of action of C.A., especially that of the zinc bound hydroxide.

In 1965 E. Breslow[32] studied the CO_2 hydration in the presence of some small peptide-Cu(II) complexes. Cu-glycylglycine (CuGG) had been previously[33] shown to dissociate a proton from a bound H_2O with a pK_a of 9.37 at 25°, and the species $CuGGOH^-$ catalyzed the hydrolysis of PNPA. k_{cat} (M^{-1} sec^{-1}) for CuGG in the CO_2 hydration at pH 9 was found to be 9.3. Equation 4 shows one of the mechanisms that was proposed for this catalyst.



(4)

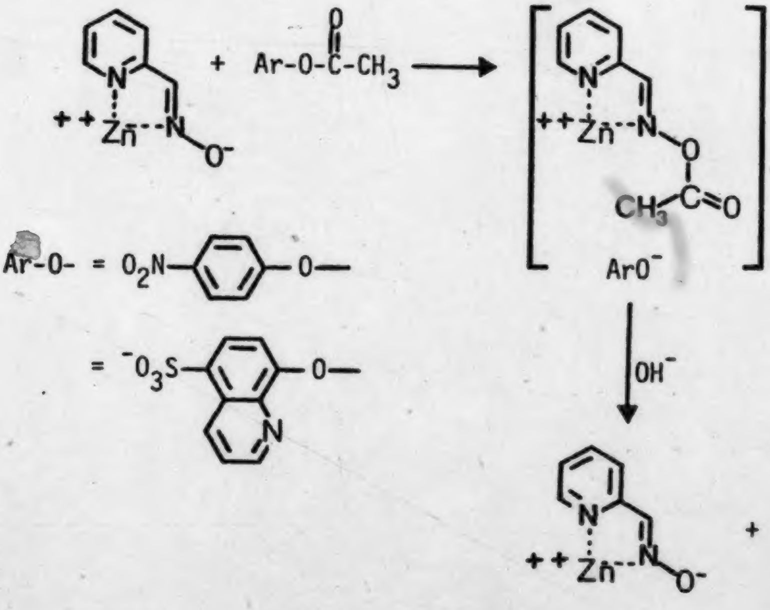
That the ionization of a bound H_2O was important catalytically was supported by the marked increase in k_{cat} from 0.11 at pH 7 to 9.3 at pH 9. Additional evidence came from the effect of added imidazole, which formed a 1:1 mixed complex with CuGG. The value of k_{cat} for this complex was less than 0.3 at pH 9. Thus imidazole led either to a direct displacement of bound OH^- or to an increase in the pK_a of bound H_2O.

The conclusion of the work was, "that a ligand on a metal ion particularly an OH^-, might participate in the enzymatic reaction."

The analogous Zn complexes were not studied because of weaker binding; CO_2 displaced the Zn from glycylglycine, for example because of carbamate formation.

The main arguments against the zinc-bound hydroxide mechanism have been twofold. First, that a zinc-bound water cannot have such a low pK_a as 7[8a,34]; second, that a zinc-bound hydroxide ion would be so polarized by the metal as to lose most of its negative charge to the zinc and thus be too weak a nucleophile to attack, for example CO_2[27].

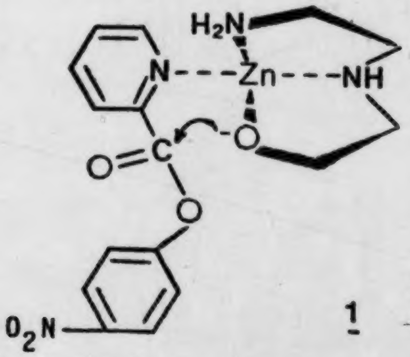
By using the zinc ion-pyridine-2-carboxaldoxime anion [Zn(II)-PCA] complex as a model, Breslow and Chipman[1d,35] provided some evidence suggesting that both arguments might be wrong. [Zn(II)-PCA] was shown to be an effective and specific nucleophilic catalyst in the hydrolysis of both PNPA and 8-acetoxyquinoline-5-sulfonate (equation 5).



(5)

Coordination to zinc ion perturbed the pK_a of PCA from 10.04 to 6.5, although a nucleophilicity comparable to hydroxide was retained[35].

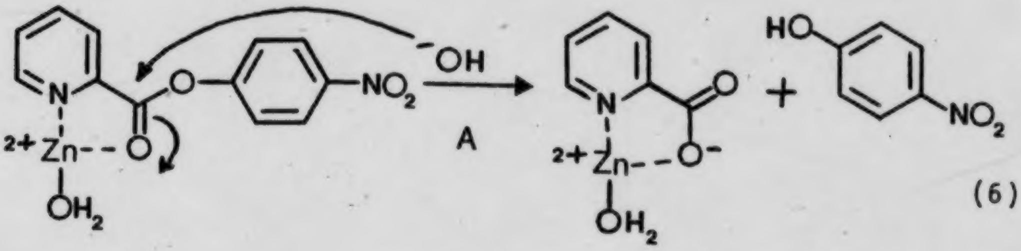
The transesterification of N-(β-hydroxyethyl)-ethylenediamine by p-nitrophenyl picolinate was shown to be subject to zinc ion catalysis by Sigman and Jorgensen[1d,36] in 1972. Their investigations indicated that reaction very probably occurred through the formation of a ternary complex (1) in which zinc ion functioned both to lower the pK_a of the hydroxyethyl moiety, and to serve as a template for the reaction.



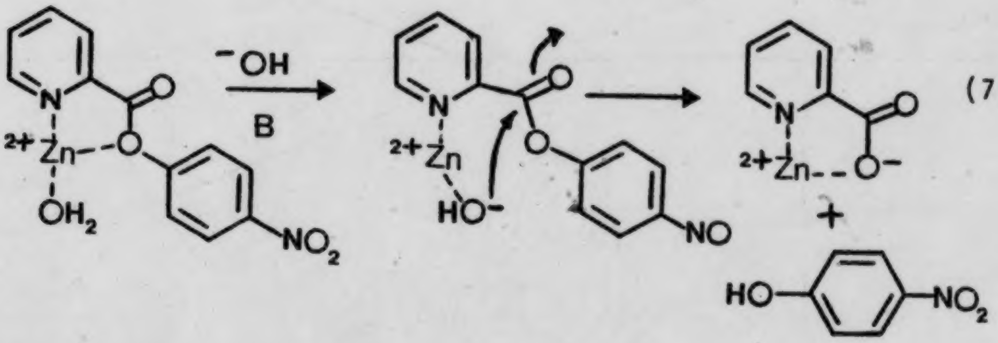
1

The pK_a of the Zn(II)-complexed β-hydroxyethyl moiety, as estimated from the pH-dependence of the observed rate of transesterification (assuming that the reaction was subject to a specific base-catalyzed reaction) was approximately 8.4, corresponding to an estimated pK_a perturbation of 3-4 units relative to the pK_a of the free hydroxyethyl group.

The last two examples demonstrated the participation of zinc-coordinated nucleophiles in a chemical transformation. It is harder to demonstrate a similar role for a water molecule coordinated in a labile complex in aqueous medium, due to the kinetic equivalence of the two mechanisms A (equation 6) and B[37a] (equation 7). *p*-Nitrophenyl picolinate is used as an example.

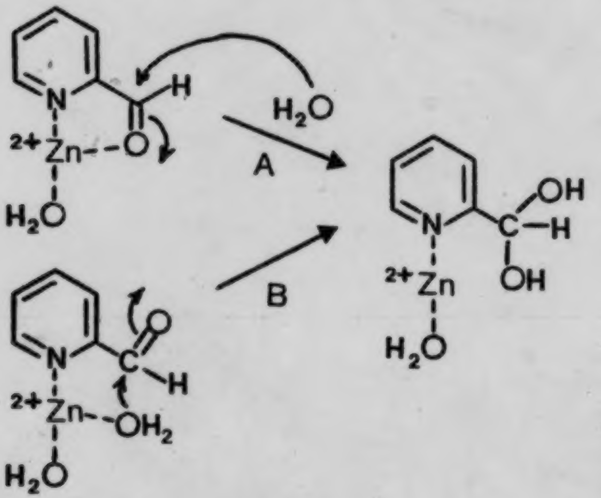


(6)



(7)

The same difficulty was encountered by Pocker and Meany[37b] when they studied the metal ion catalyzed hydration of 2- and 4-pyridine carboxaldehydes (2-PA and 4-PA). The catalysis afforded by metal ions was shown to be several orders of magnitude bigger for 2-PA than for 4-PA. They attributed this difference in catalytic behavior to the special arrangement of the ring nitrogen and aldehydic group in 2-PA which is absent in 4-PA. However, they were not able to "differentiate between a hydration arising from reaction schemes involving an attack by external water on the metal-2-PA complex (A) and one in which the attacking water itself comes from the metal hydrate (B) because of the labile nature of these complexes" (equation 8).

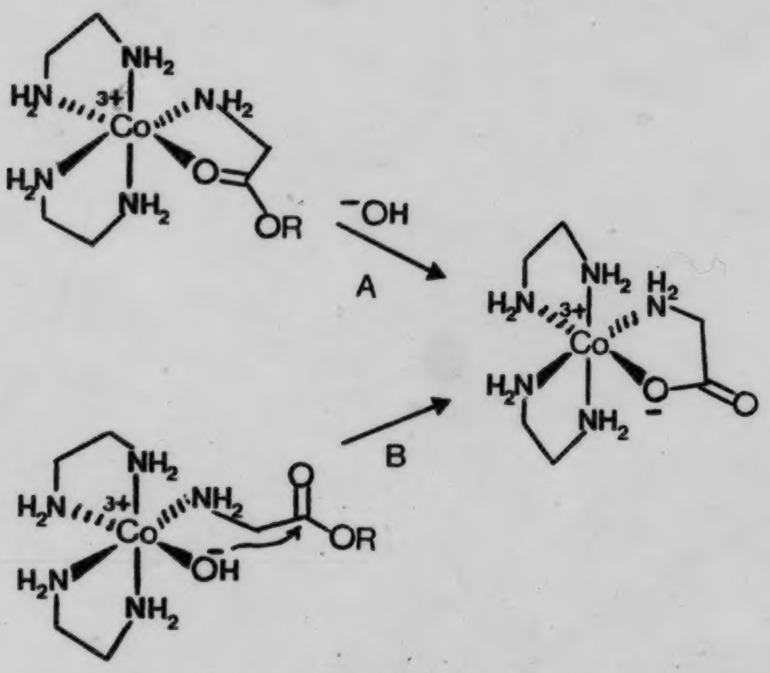


(8)

Since C.A. is equally active in the hydration of both aldehydes[37b], they concluded that no pyridine nitrogen-to-metal coordination is involved in the enzymatic hydration of 2-PA or 4-PA.

With the use of inert M(III) transition metal complexes, it is possible to demonstrate the participation of metal-bound hydroxide in a chemical transformation.

Buckingham et al[1d,38] concluded from their ^{18}O-labeling experiments that the Co(III)-catalyzed hydrolysis of glycine esters proceeded by dual pathways at approximately equal rates by mechanisms corresponding to A and B (equation 9).



(9)

Water coordinated to Co(III) was shown to be an effective nucleophile (pK_a ~ 6) in the hydrolysis of glycine esters.

Chaffee et al[39a], and Palmer and Harris[39b] found that Co(III)-, Rh(III)-, and Ir(III)-coordinated hydroxide was an effective enough nucleophile to react with CO_2. They measured the rate of CO_2 uptake by hydroxopentaamminemetal(III) ion to form carbonatopentaamminemetal(III) ion, according to equation 10.

$$[M(NH_3)_5OH]^{++} + CO_2 \overset{k}{\rightleftharpoons} [M(NH_3)_5CO_3H]^{++} \rightleftharpoons [M(NH_3)_5CO_3]^{+} + H^{+} \qquad (10)$$

The second order rate constants (k, M^{-1} sec^{-1}) were determined to be 220, 420, and 590 for Co, Rh, and Ir respectively. The respective pK_a's were: 6.6, 6.8, and 6.7. As pointed out by the authors[39a] it is significant to compare the reactivities to carbon dioxide of water, hydroxopentaamminometal(III) ion and hydroxyl ion, and their relative basicities. The latter are in a ratio of approximately $1:10^7:10^{16}$. The rate constants do fall in this order, but obviously not in any simple proportion, since while OH^- converts CO_2 to carbonate 10^7 times more rapidly than does water, it is only 20-40 times more effective than the M(III) complexes.

In 1974 Appleton and Sarkar[40] titrated some imidazole-Zn(II) complexes and concluded that the pyrrole hydrogen of a neutral imidazole moiety may be induced to ionize at pH's near 7 by coordination of the pyridine nitrogen to a zinc ion. Analysis of the Zn(II)-N-methylimidazole system yielded a value of 9.1 for the pK_a of a metal-bound water molecule in the presence of a triimidazole ligand field. However these results were complicated by the formation of precipitates.

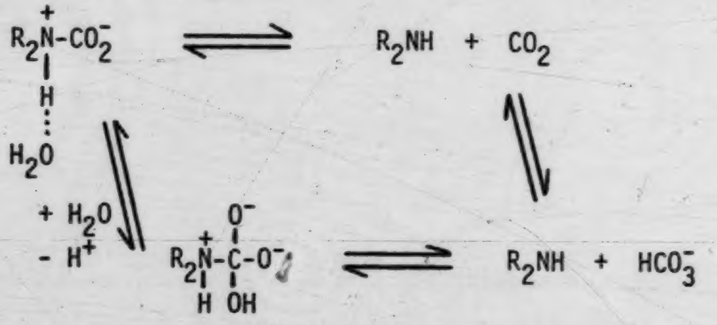
Martin[41a] criticized heavily these results saying that they were observed under non-equilibrium conditions and he found no precedent in the literature for such a pronounced acidification in imidazole compounds by zinc ion.

In agreement with Martin[41a], Sóvágó et al[41b] using nmr techniques attributed the deprotonation of the histamine-Zn(II) complex starting at pH ~ 8 to a zinc-bound H_2O and not to an imidazole pyrrolic NH.

To evaluate the "zinc-imidazolate" mechanism in a model system, Sargeson et al[42a] compared the effect of $[Co(NH_3)_5OH]^{++}$ and $[Co(NH_3)_5Im]^{++}$ (Im = imidazolate ion) on PNPA. In water, the hydroxide complex is 6 x 10^3 times less reactive than the imidazolate complex, the coordinated imidazolate being of similar nucleophilic reactivity to free OH^- towards PNPA. It was concluded[1h] that these results support a mechanism for the esterase activity of C.A. whereby a zinc-imidazolate attacks the carbonyl of ester substrates, giving an intermediate acylhistidine, which is then cleaved by water. Coordination of N-acetylimidazole to Co(III) was found to enhance the rate of hydrolysis by ca. 20-fold relative to free N-acetylimidazole. However, direct attack of Zn-Im on CO_2 in C.A. was recognized as unlikely because the resulting carbamate would decompose by heterolytic N-C fission rather than H_2O addition followed by elimination of HCO_3^- or H_2CO_3. Sargeson based this argument on previous carbamate decomposition studies[42b,c]. Co(III)-bound carbamates decompose in acidic media without oxygen exchange from water to CO_2[42b] (equation 11).

$$[Co(NH_3)_5OCONH_2]^{++} \xrightarrow[H_2O^*]{H^+,\ NaNO_2} [Co(NH_3)_5O^*H_2]^{+++} + CO_2 + N_2 \qquad (11)$$

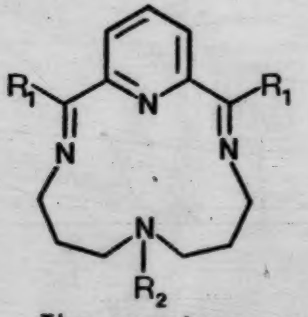
However, it is not possible at neutral pH's to discard the possibility by which water adds to the carbamate prior to the N-C bond cleavage[42c] (equation 12)



(12)

Sargeson suggested that C.A. might operate via two mechanisms: Zn-OH for CO_2, but Zn-Im for esters.

A model supporting the Zn-OH mechanism was provided by Woolley[31], who prepared Zn(II) and Co(II) complexes of the ligands 2(a-c).



2a R_1=Me R_2=H

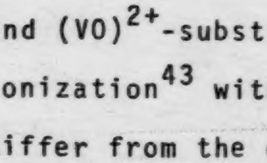
2b $R_1=R_2$=H

2c $R_1=R_2$=Me

(CR)

The complexes are five coordinate, with 4 nitrogen donors from 2 and water as the fifth ligand. Titration of the zinc:2(a-c) complexes yielded pK_a values of 8.69, 8.12 and 8.13 respectively, at 25°, while that for $[CoCR]^{++}$ was found to be about 8. Six coordinated Ni(II) and Cu(II) complexes showed pK_a's > 11. $[ZnCR\cdot OH]^+$ was found to be an efficient catalyst for acetaldehyde hydration, comparable to the enzyme, but a very modest one for CO_2 hydration. The conclusion from these experiments was that the metal bound hydroxide would have sufficient nucleophilic power to account for the enzyme's activity in acetaldehyde hydration, although not in CO_2 hydration, if the Zn environment was the same in the enzyme as in CR complexes. However, a poorly solvated nucleophile would, in a non-polar environment be more reactive[42]. Some inactive Cu^{2+}- and $(VO)^{2+}$-substituted carbonic anhydrases show an ionization[43] with $pK_a \sim 7$. If the active site did not differ from the one in the Zn^{2+}-enzyme (not very likely), it must be shown that the ionization does not correspond to metal-bound H_2O before the Zn-OH mechanism can be accepted.

While this thesis work was in progress, Breslow et al[44] reported the synthesis and preliminary physical studies of some molecules containing three chelating imidazole rings: *tris*-(2-imidazolyl) carbinol (2-TIC) 3, and *tris*-[4(5)-imidazolyl] carbinol (4-TIC) 4.



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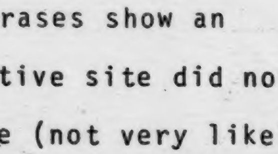
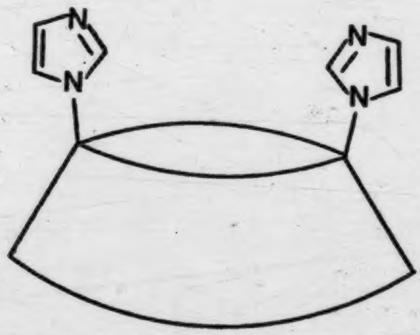


4

4-TIC was found to be a very good metal binder, comparable to C.A. for Co(II), Ni(II) and Cu(II), but about 100 times worse binder for Zn(II). Also, the Co(II) complex of 4-TIC did not have the blue color characteristic of tetracoordinate Co(II). They suggested that these ligands were a bit too small, so that octahedral complexing was facile, and that a somewhat larger ligand related to 4-TIC might well mimic better both the spectroscopic behavior of C.A. and also the extraordinary Zn(II) affinity of the enzyme.

Recently Tabushi et al[45] prepared C.A. models (5 and 6) using cyclodextrin.



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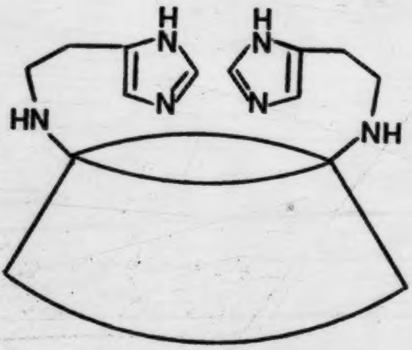


6

The complexes $ZnCl_2$:6 and $ZnCl_2$:5 catalyzed (k_{cat} = 166 and 16.2 M^{-1} sec^{-1} respectively) the hydration of CO_2 (pH = 7.50, 25°).

By comparing the rate enhancements of these cyclodextrin derivatives with those from $(imidazole)_2Zn(II)$ and $(histamine)_2Zn(II)$ they concluded that both the hydrophobic environment provided by cyclodextrin and the zinc bound to the imidazoles contributed to give some of the C.A. activity. Another interesting finding was that the introduction of additional base enhanced the activity, as seen for $ZnCl_2$:6 compared to $ZnCl_2$:5. Although not mentioned by the authors, this rate enhancement of $ZnCl_2$:6 vs. $ZnCl_2$:5 could have been due simply to different coordination geometry around the zinc ion, and not necessarily to the presence of additional bases.

Finally, and although it does not fit into the category of a model, it is worthwhile to mention the work of Kaiser et al[46] who produced and characterized a 36-aminoacid residue peptide containing the metal-binding ligands at the active site of HCAB. Due to precipitation problems they were not able to study the Zn(II):peptide complex. Instead they used Co(II). Studies on the hydrolysis of 5-nitro-2-hydroxy-α-toluenesulfonic acid sultone[16d] and hydration of CO_2, showed that the peptide in the presence or absence of Co(II) had little, if any, catalytic activity. They attributed this lack of success to the poor binding ability of the peptide, and reached the conclusion that the next step in designing a C.A. model should be the preparation of a peptide capable of binding the active site metal ion more tightly.

II. RESULTS AND DISCUSSION

A. INTRODUCTION TO THE PRESENT WORK

The models studied so far, although successful in some aspects, all suffer from some problems. For example, E. Breslow[32] demonstrated that OH^- bound to Cu(II) was a modest catalyst for CO_2 hydration, but did not consider the situation for Zn(II). R. Breslow et al[35] and Sigman et al[36] showed the participation of zinc-coordinated nucleophiles in ester hydrolyses, but these nucleophiles have no direct analogy in the enzyme. Results obtained from models involving M(III) complexes[38,39,42] can not be directly extrapolated to the analogous Zn(II) complexes, due to the different ionic charge and to the very different reactivity in terms of ligand exchange. Woolley[31] proved that a zinc bound water can have low pK_a (~ 8.1) and be an effective catalyst for acetaldehyde hydration. However, the number and nature of the nitrogens coordinating the metal have little in common with the enzyme. A similar problem is encountered with the model of Tabushi et al[45], where only two imidazoles are bound to the metal, compared to three in the active site of C.A. In this case little can be said about the mode of action of the model catalyst.

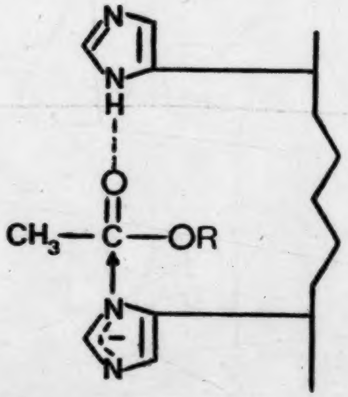
The purpose of this work was to build models to mimic the maximum possible number of physicochemical features of the active site of C.A. (three imidazole ligands surrounding the metal, pseudotetrahedral arrangement, ionizable group with $pK_a \sim 7$, blue Co(II) complex, slow Zn(II) binding rate, etc.) and see how well they could reproduce the catalytic behaviour of the enzyme.

If a model and the enzyme have a lot in common, the extrapolation of some property from the model to the enzyme is safer than if they are only vaguely related. From this point on, our definition of model will be much narrower than Woolley's[31]. The "ideal model" will be the simplest chemical system which mimics the maximum possible number of properties of the enzyme.

B. POLYMERS

Our first attempt at making a model for C.A. was one which in retrospect was ambitious in the sense that we relied upon a polymeric catalyst incorporating pendant imidazoles which were hoped to bind the metal in a more or less correct geometry.

If one incorporates catalytically active functional groups in synthetic polymers, the apparent catalytic activity may be enhanced due to association between catalyst and substrate[47] and to some cooperative effects between different catalytic centers in the polymer[48]. For example, poly-4(5)-vinylimidazole was found to be better catalyst than imidazole for the hydrolysis of nitrophenolacetates[48b]. For the negatively charged substrate 4-acetoxy-3-nitrobenzoic acid a bell-shaped pH-rate profile was observed as a result of electrostatic attraction to the protonated sites of the polymer. The enhanced catalytic activity towards the neutral substrate PNPA was attributed to the cooperative participation of the anionic forms of the pendent imidazole groups. Two possible mechanisms were postulated. One is that the neutral imidazole group assists the attack of anionic imidazole on the ester carbonyl (7) and the other is that the neutral imidazole attack on the ester gives a tetrahedral intermediate which is then attacked by an anionic imidazole acting as a general base (8).



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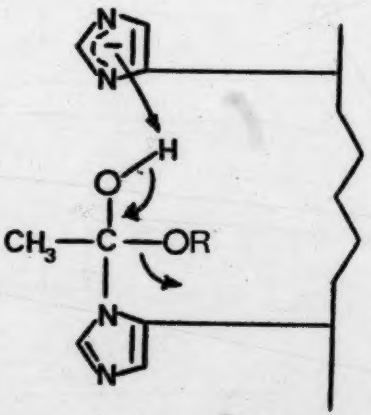
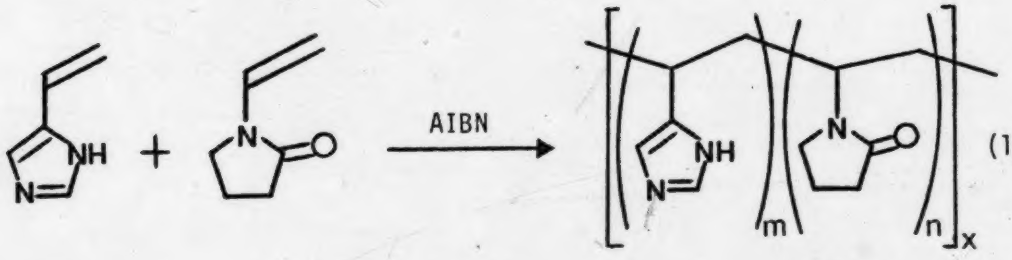


8

With these ideas in mind copoly[N-vinylpyrrolidinone-4(5)-vinylimidazole] was prepared by free radical polymerization of a mixture of N-vinylpyrrolidinone and 4(5)-vinylimidazole (equation 13).



(13)

If all events went optimally the imidazole residues would bind zinc perhaps in the correct geometry, the polymer chain would provide some hydrophobic environment, and the pyrrolidinone residues would be like the peptide linkages of a protein, thus affording a macromolecule with at least some centers which could be similar to the active site of C.A.

The imidazole content of the copolymer was determined from the N and O microanalyses. The number of moles of imidazole per 100 g of polymer (n_I) is given by the expression:

$$n_I = [\%\ N - (\%\ O/16) \times 14]/28 \qquad (14)$$

where % N and % O are the percentages of nitrogen and oxygen in the polymer respectively.

The polymer was complexed with Zn(II) in the ratio [Imidazole]/[Zn(II)] = 3, and tested for catalytic activity towards PNPA hydrolysis and CO_2 hydration. No catalytic rate enhancement was observed in any case.

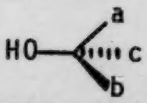
This fact, and the inability to assign the nature of the polymer:Zn(II) complex induced us to discontinue this work, and study simpler, easier to characterize, models.

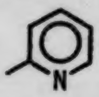
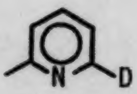
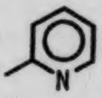
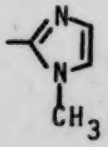
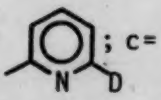
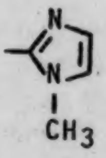
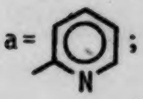
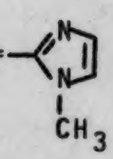
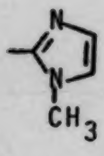
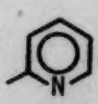
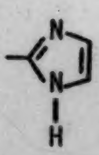
C. CARBINOLS

These models were designed to bind metals in a tridentate fashion, similar to the way Zn(II) is bound in the active site of C.A. Several features had to be present in the molecules before they could be considered good candidates for C.A. models. The first one was M(II) binding ability. The check this, the binding constants with several divalent cations were determined. Since the pK_a's were needed as data for obtaining the metal binding constants, they were also determined. Second, in order to check that the mode of binding was tridentate, nmr experiments of ligand solutions with varying Zn(II) concentrations were performed. Thirdly, since the coordination number around the metal should be four to approximate the pseudotetrahedral binding in C.A.[4,5], the U.V. visible spectra of the ligands using Co(II) as a probe[8] were recorded. Finally, the zinc complexes were titrated with base in order to observe the titration of a group with pK_a ~ 7, the basic form of which in C.A. is associated with activity[1a,1c,1e]. Table III shows ligands 9-17 which for synthetic convenience were prepared as carbinol derivatives. They contain pyridine and several substituted imidazole moieties to compare their different behaviour in terms of metal binding.

TABLE III

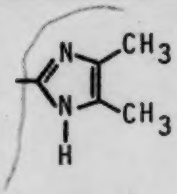
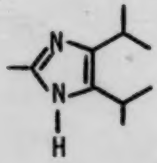
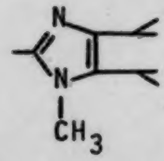
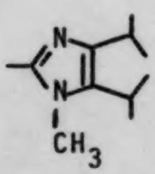
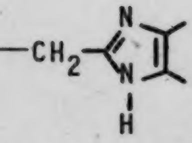
Compounds 9 to 17.



Compounds	Substituents
9	a=b=c= 
d-9	a=b=c= 
10	a=b= ; c= 
d-10	a=b= ; c= 
11	a= ; b=c= 
12	a=b=c= 
13	a=b= ; c= 

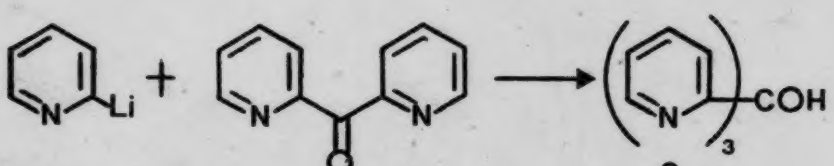
continued...

Table III (continued)

Compound	Substituents
14	a=b=c= 
15	a=b=c= 
16	a=b=c= 
17	a=b=  ; c= 

1. SYNTHESES

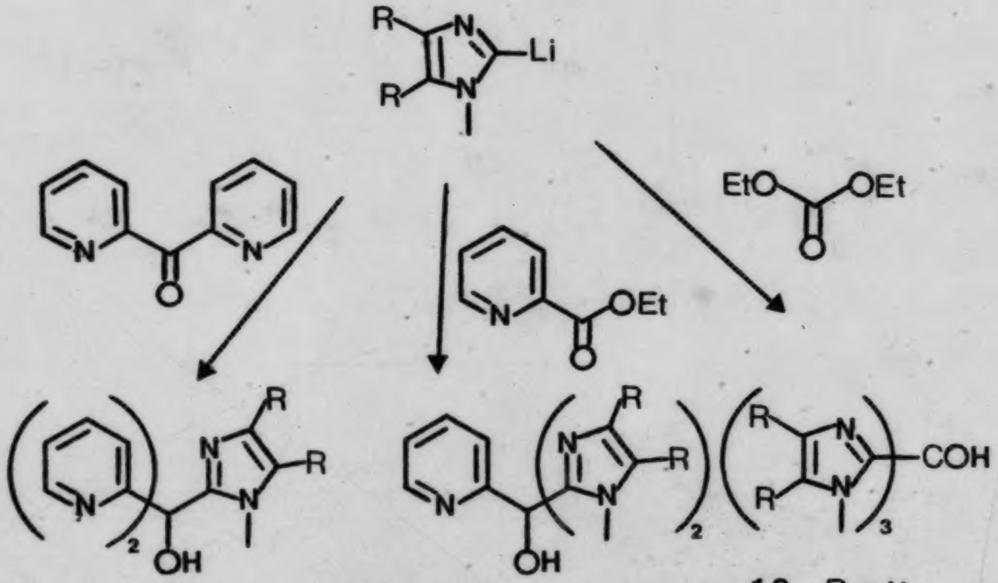
Compounds 9-16 were synthesized by reacting the metalated derivatives of 2-bromopyridine or the appropriate N-substituted imidazoles according to the methods of Wibaut et al[50] (equation 15), Roe[51a], and Shirley and Alley[51b] (Scheme X), with the appropriate carbonyl compounds.



9

(15)

SCHEME X


R
R
N
N
Li
EtO
OEt
O
N
N
O
N
OEt
O
R
N
R
N
N
2
OH
R
N
R
N
N
2
OH
R
N
N
R
COH
3

10 R=H

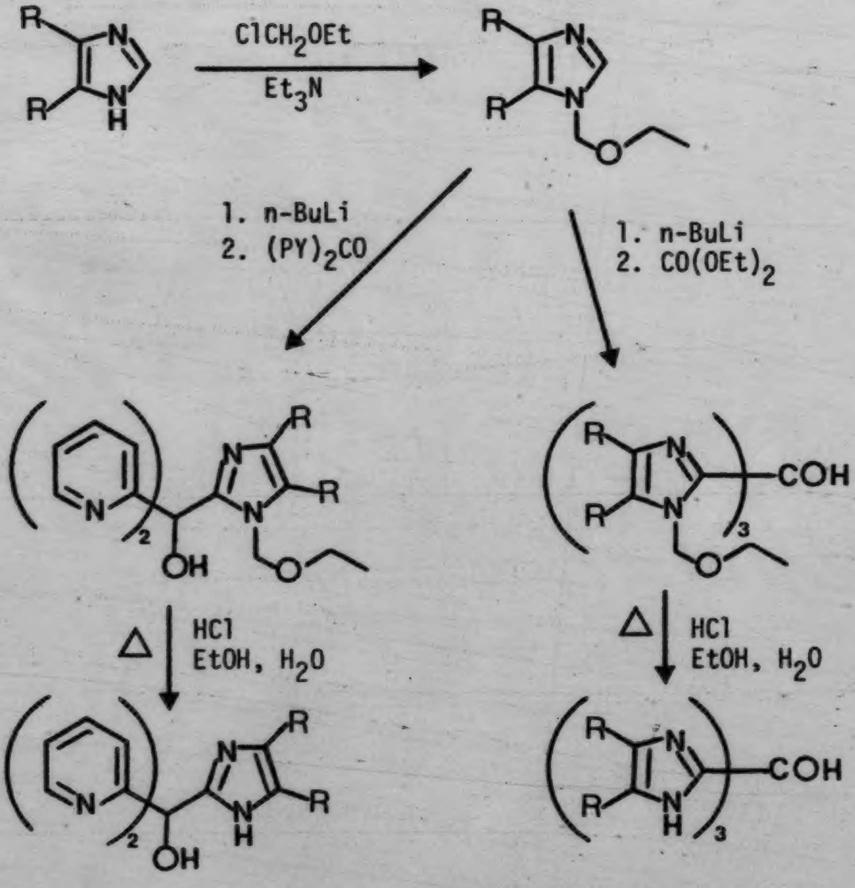
11 R=H

12 R=H

16 R=i-Pr

When the imidazole moiety was required to have free N-H in the final product, the starting imidazole was N-protected before metalation. This was done according to the methods of Roe[51a] and Tang et al[44], using an ethoxy- or methoxymethyl protecting group, which was removed[44] by reflux in hydrochloric acid (Scheme XI).

SCHEME XI


$ClCH_2OEt$
Et_3N
1. n-BuLi
2. $(PY)_2CO$
1. n-BuLi
2. $CO(OEt)_2$
△ HCl
EtOH, H_2O
△ HCl
EtOH, H_2O

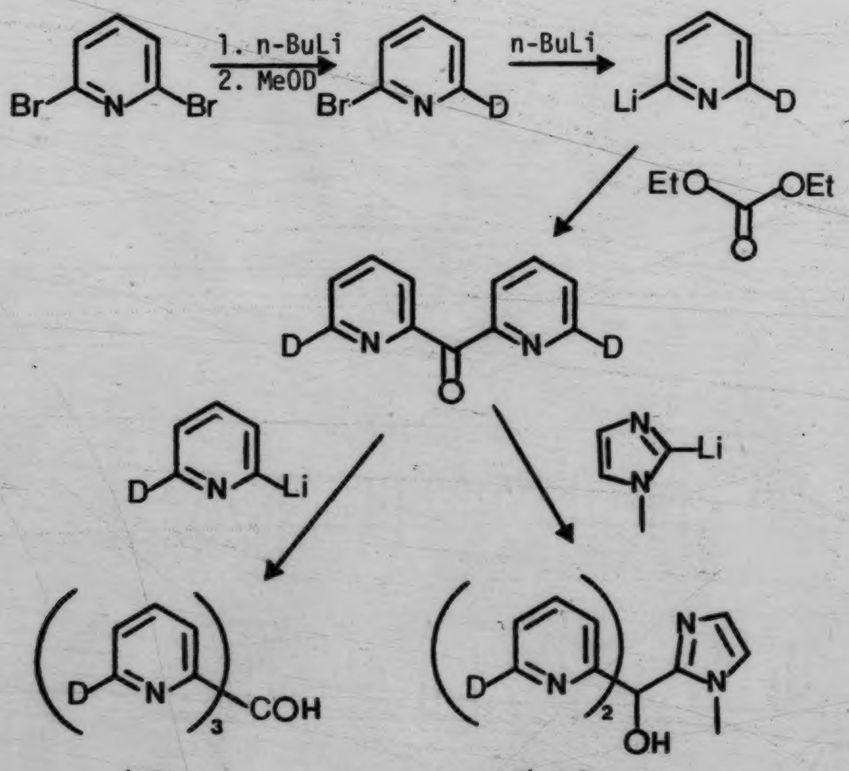
13 R=H

14 R= Me

15 R= i-Pr

The deuterated compounds d-9 and d-10 were prepared using 2-bromopyridine-6d as a starting material. This was obtained by deuteration of 2-lithio-6-bromopyridine prepared according to Gilman's procedure[52] (Scheme XII).

SCHEME XII


Br
N
Br
1. n-BuLi
2. MeOD
Br
N
D
n-BuLi
Li
N
D
EtO
OEt
O
D
N
O
N
D
D
N
Li
N
Li
N
D
N
COH
3
D
N
2
OH
N
N

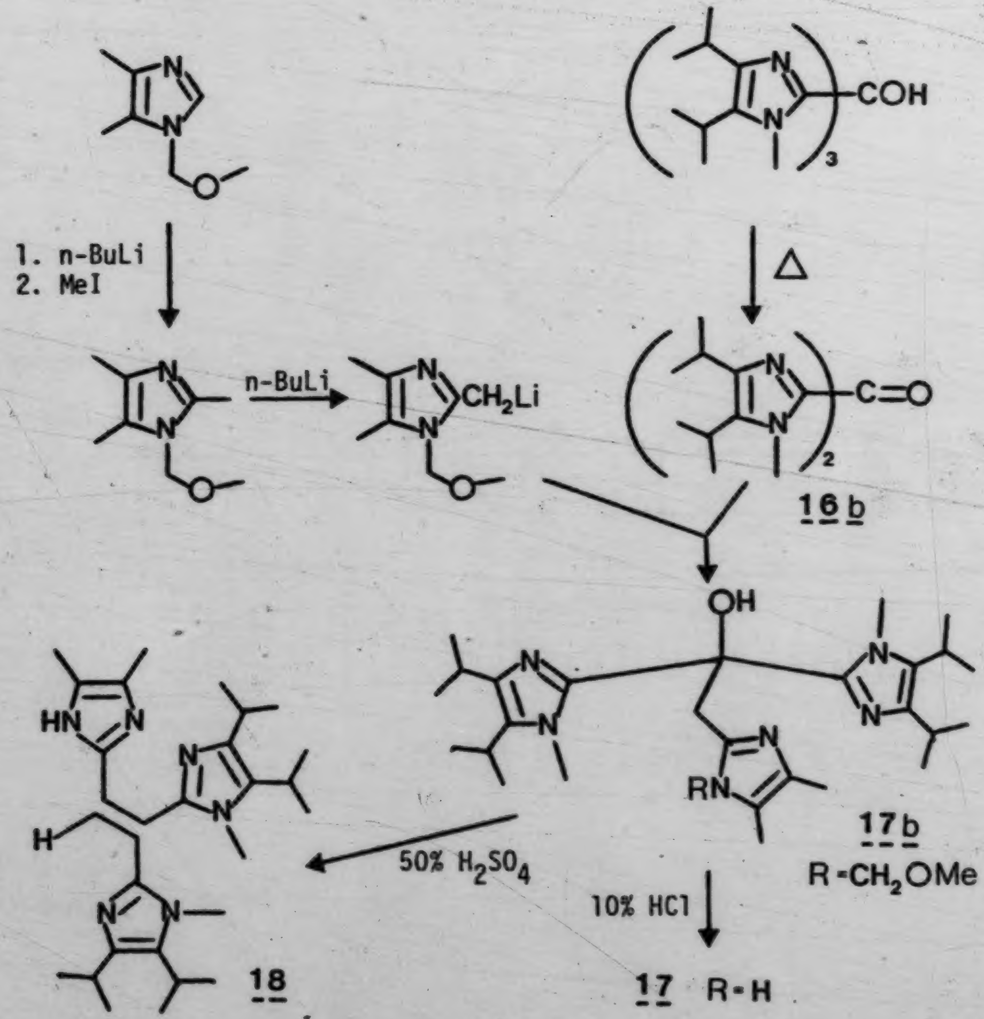
d-9

d-10

Compound 17 was synthesized using lithiated N-protected-2,4,5-trimethylimidazole as a nucleophile (Scheme XIII). It is worth noting the exceptionally long time (2 weeks) required to deprotect 17b in refluxing 10% aq. HCl.

Higher acid concentrations gave substantial yields of dehydrated product 18, as evidenced by a very intense M^+ peak in the mass spectrum and the presence of an olefinic singlet at δ 7.0 ppm in the nmr spectrum* of the hydrolized product.

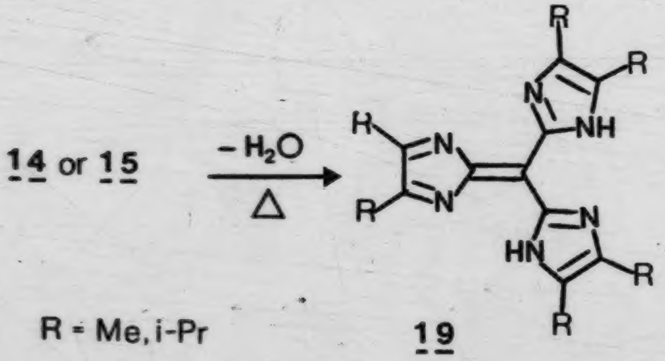
SCHEME XIII


1. n-BuLi
2. MeI
n-BuLi
CH₂Li
COH
3
Δ
C=O
2
16b
OH
17b
R=CH₂OMe
50% H₂SO₄
10% HCl
HN
18
17 R-H

---

[*] δ_H estimated[53a] for $Ar_2C{=}CHAr$ is 6.90 ppm

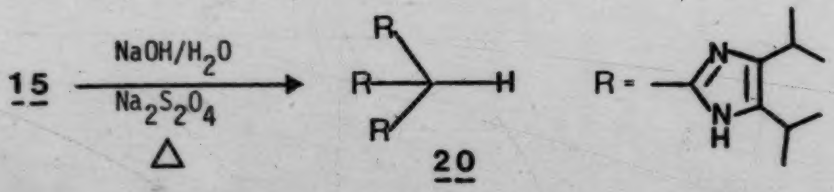
Ligands 14 and 15 upon drying (for microanalytical purposes) tended to dehydrate (as evidenced by the build-up of an M^+-H_2O peak in the mass spectrum) to form intensely colored crystals which appeared to possess extensive conjugation as might be expected for a fulvene-like 19 (equation 16).


14 or 15
– H2O
Δ
H
N
N
R
N
NH
HN
N
R
R = Me, i-Pr
19

(16)

^{1}H-nmr experiments on these crystals in $CDCl_3$ showed complete molecular symmetry as expected if the alkyl groups were tautomerically equivalent on the nmr time-scale.

Since 15 proved to be inappropriate for study the synthesis of ligand 20 was attempted according to equation 17.


15
NaOH/H2O
Na2S2O4
Δ
R
R
H
R
20
R =
N
N
H

(17)

The corresponding methane was present as evidenced by a ^{1}H-nmr singlet at δ 5.4*, and the material appeared reasonably stable as its HCl salt. Unfortunately in basic media, the methine H proved extremely susceptible to air oxidation[53c] which converted it back to 15 which again suffered deleterious dehydration to form what we believe is 19 (equation 16).

2. BASICITIES

Ionization constants of the protonated ligands were determined by potentiometric titration methods outlined by Albert and Serjeant[54a] and Rossotti and Rossotti[54b]. Data were analyzed by a modified computer version of the Simm's method[55] (APPENDIX I). The pK_a's obtained are listed in Table IV.

There is no predictable trend in the basicities for 9-12 although on the average the N-methylimidazoles are more basic. Where comparison can be made between those ligands containing N-CH_3 and N-H imidazoles (i.e. 10 and 13; 12 and 2-TIC[44]) the latter appear to be more basic at least at pK_{a1} and pK_{a2}. The pK_a's of the 4,5-disubstituted imidazoles were determined in increasingly organic media for solubility reasons and therefore cannot rigorously be compared with those values determined in water. As can be seen from the two sets of data re-

---

*δ_H for $(C_6H_5)_3CH$ is 5.6 ppm[53b].

TABLE IV

pK_a's for ligands 9-17 determined by potentiometric titration[a].

Ligand	pK_{a3}	pK_{a2}	pK_{a1}
9	1.5	2.1	4.7
10	1.5	2.1	5.4
11	1.6	2.65	5.6
12	1.7	2.5	5.5
13[b]	1.44±0.04	2.87±0.1	6.46±0.05
14[c]	2.85±0.03	5.12±0.03	7.54±0.03
14[d]	2.58±0.02	4.78±0.02	7.40±0.02
15[d]	2.33±0.04	4.49±0.03	—[e]
16[f]	<1.5	3.80±0.05	6.90±0.05
17[g]	2.45±0.05	4.7±0.1	8.2±0.2

[a] Generally determined in solution comprised of 3.0 mL 0.25 $\bar{M}$ KNO_3, 1.0 mL 0.025 $\bar{M}$ ligand and either 0.50 or 1.00 mL 0.1095 $\bar{M}$ HNO_3, and have a ± 0.1 unit precision unless noted.

[b] 3 mL (1:1 acetone/H_2O), 0.25 $\bar{M}$ KNO_3, 1.0 mL of 0.025 $\bar{M}$ ligand in acetone, and 1.0 mL of 0.1095 $\bar{M}$ HNO_3.

[c] Determined in 3 mL, 0.25 $\bar{M}$ KNO_3, 1.0 mL of 0.025 $\bar{M}$ ligand containing 0.1095 millimoles of HNO_3.

[d] 3 mL of 1:1 acetone/H_2O, 0.25 $\bar{M}$ KNO_3, 1.0 mL of ligand (0.025 $\bar{M}$) containing 0.1095 millimoles HNO_3.

[e] pK_1 could not be determined due to precipitation in this medium.

.......continued

Table IV (continued)

[f] 3 mL of 75:25 acetonitrile/H_2O, 0.25 $\bar{M}$ KNO_3, 1.0 mL of 0.1042 $\bar{M}$ HNO_3, 1.0 mL of 0.0269 $\bar{M}$ ligand in ethanol.

[g] 3 mL of 1:1 acetone-H_2O, 0.25 $\bar{M}$ in KNO_3; 1.0 mL of 0.0245 $\bar{M}$ ligand in methanol, 1.0 mL of 0.1042 $\bar{M}$ HNO_3.

ported for 14, in the more organic environment the pK_a's are lower, consistent with expectations based on solvent polarity, and clearly 4,5-substitution (16 and 12, 14 and 2-TIC[44]) increases the basicity as expected.*

Ligand 17, in which the 4,5-dimethylimidazole is extended from the carbinol centre by a methylene unit, is the most basic particularly at pK_{a2} and pK_{a1}, when compared with 14, 15, or 16. Presumably this is due to increased separation of the positive charges upon protonation[44,57].

3. METAL BINDING CONSTANTS

These were determined by titration of the ligands in the presence of metals according to the method of Rossotti and Rossotti[54b]. The data were analyzed by a computer program[*] (APPENDIX II) and the results are shown in Table V.

---

*Whereas 2-methylimidazole has a pK_a of 7.85, that of 2,4,5-trimethylimidazole is 8.92[56].

The general ordering for metal stability constants for 9-14 is $Zn^{++} \leq Co^{++} < Ni^{++} < Cu^{++}$ and a first and second binding constant indicative of a 1:1 ($L:M^{++}$) and 2:1 ($L:M^{++}:L$) complex was obtained for each. Ligands 15 and 16 with 4,5-di*iso*propyl substituents appear from space-filling models to encapsulate the metal sufficiently to inhibit 2:1 complexation and no second binding constants were observed for these. It is worthy to note that for C.A. the order of stability constants is $Cu^{++} > Zn^{++} > Ni^{++} > Co^{++}$ at pH 5.5[8b], and the metal-ligand geometry is demonstrated to be distorted tetrahedral at least for Zn^{++}[4,5] and Co^{++}[7b,10]. While we have no details for the coordination numbers for 9-14: M^{++} complexes other than those implied from UV studies with Co^{++} (see below), 15, 16, and 17 appear from models only to allow tetrahedral geometry if they are bound symmetrically. It would appear from Table V that Zn^{++} is bound to 15 and 17 stronger than is Co^{++} perhaps as a result of the enforced tetrahedral geometry[58].

The data for 16 indicate that Zn^{++} and Co^{++} binding

---

[*] The basic computer version was kindly supplied by Prof. R. Breslow of Columbia University and was modified to be utilized for ligands which possess only two pK_a's.

TABLE V

Metal stability constants for ligands 9-17[a,b].

Ligand	Parameter	Value			
		Zn^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}
9	pK_1	5.4	6.4	6.2	6.6
	pK_2	4.9	5.1	5.7	6.35
10	pK_1	5.9	6.6	7.1	7.5
	pK_2	5.1	6.2	6.6	7.0
11	pK_1	5.0	5.2	6.8	7.2
	pK_2	4.3	4.5	6.2	5.6
12	pK_1	3.15	-	4.8	-
	pK_2	2.7	-	3.5	-
13	pK_1	7.50±0.05	8.67±0.01	-	>10.5
	pK_2	6.67±0.17	8.10±0.10	-	>10
14[c]	pK_1[d]	8.86±0.10	9.46±0.07	-	11.91±0.06
14[e]	pK_1	8.81±0.03	9.65±0.03	9.90±0.05	11.41±0.03
	pK_2	7.53±0.03	8.93±0.04	8.70±0.15	9.64±0.7
15[f]	pK_1	7.50±0.04	<7.0	ppte	9.28±0.2
16[g]	pK_1	4.0±0.1	4.0±0.1	-	-
17[h]	pK_1	6.0	4.3	4.5	7.7
Human CAB[i]	pK_1	10.5	7.2	9.5	11.6

.......continued

Table V (continued)

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[a] 25.0°C, 3.0 mL of 0.25 $\bar{M}$ KNO_3, 1.0 mL of 0.025 $\bar{M}$ ligand containing 0.1095 millimoles HNO_3, 0.5 mL of 0.011 $\bar{M}$ metal.

[b] Precision of ±0.1 units unless noted.

[c] Determined as in a.

[d] Precipitation prevented pK_2 determination.

[e] 3.0 mL (1:1 acetone/H_2O) of 0.25 $\bar{M}$ KNO_3, 1.0 mL of 0.025 $\bar{M}$ ligand in 0.1095 $\bar{M}$ HNO_3, and 0.5 mL of 0.011 $\bar{M}$ metal.

[f] 3.0 mL (1;L acetone/H_2O) of 0.25 $\bar{M}$ KNO_3, 1.0 mL of 0.025 $\bar{M}$ ligand in acetone, 1.0 mL of 0.1095 $\bar{M}$ HNO_3, and 0.5 mL of 0.011 $\bar{M}$ metal.

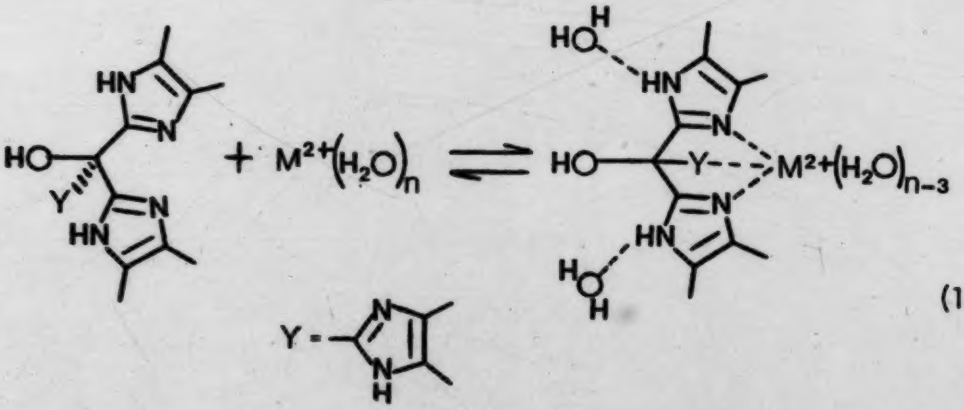
[g] 3.0 mL (75:25 CH_3CN/H_2O) 0.25 $\bar{M}$ KNO_3, 1.0 mL of 0.0269 $\bar{M}$ ligand in ethanol, 0.5 mL of 0.1042 $\bar{M}$ HNO_3, 0.5 mL of 0.11 $\bar{M}$ metal.

[h] 3 mL 1:1 acetone-H_2O.

[i] Reference 8b.

are equivalently weak. Finally it is of note that 17 binds metal in the order $Cu^{++} > Zn^{++} > Ni^{++} > Co^{++}$ parallel to that for C.A., although the former values are about 4 pK units lower than those for the enzyme.

A pattern emerges when one considers the stability constants for N-CH_3 imidazole derivatives 10, 12, and 16 when compared to their N-H imidazole analogues 13, 2-TIC[44], and 15 respectively. In every case N-methylation reduces the metal binding ability by some 3-4 pK units. This observation contrasts the reports[59] in which N-methylimidazole and imidazole exhibit nearly identical pK_a and stability constant values for Cu^{++}, Cd^{++}, and Ag^{+}. While we have no unambiguous reason for the stronger binding ability of N-H vs. N-CH_3 imidazoles, the phenomenon may be related to a better solvation of the former when bound to metal as in equation 18.



(18)

4. NUCLEAR MAGNETIC RESONANCE STUDIES

^{1}H-nmr spectra of roughly 10^{-2} M solutions of ligands 9-17 were determined in D_2O as a function of increasing [$ZnBr_2$]*. After subsequent additions of Zn^{++}, the pD (pD = pH (meter reading) + 0.4)[60a] of the test

---

[*] When needed, methanol-d_4, acetone-d_6, or dimethylsulfoxide-d_6 were added to increase the solubility of the complexes.

solutions decreased due to release of protons from the ligand accompanying metal binding, and the pD of the solutions at the end of the Zn^{++} additions was in the order of 4. This is important because the potentiometric titrations to determine the zinc-binding constants invariably showed the complexes to be fully formed by pH ~ 3.5 when the pK_1 was 5 or more. Chemical shifts for ligands 9-13 and the Zn^{++} complexes for 9 and 10 are given in Table VI.

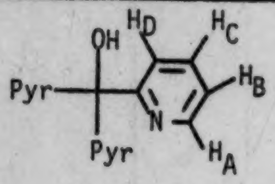
Some interesting trends that provide information about the structure of the complexes are apparent from consideration of the data for 9; the pyridine-H spectral region is illustrated in Fig. 3. Assignments are easily verified by comparing the spectra of 9 and d-9 (Fig. 4). Subsequent additions of Zn^{++} showed the build-up of an additional set of resonances at the expense of those for 9, and finally when the $9/Zn^{++}$ ratio was 2, a clean spectrum was produced indicative of a symmetrically bound 2:1 complex* (Fig. 3). Now it appears that H_D is the low field resonance at δ 8.54 coupled to H_C at δ 8.25 (J_{CD} = 7.6 Hz). By elimination H_A in the 2:1 complex is shifted upfield to δ = 7.7 and is coupled to H_B at δ 7.40 (J_{AB} = 5.4 Hz). This upfield shift probably occurs because H_A

---

* This spectrum agrees very well with the one of $Zn(9)_2(ClO_4)_2$ reported recently by Boggess and Boberg[60b].

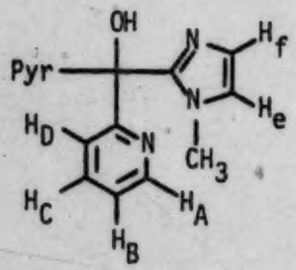
TABLE VI

Chemical shift values and assignments for ligands 9-13 and their Zn^{2+} complexes determined in D_2O solution.

Ligand	δ and J value			
	H_A	H_B	H_C	H_D
9 	8.58 ddd J_{AB} = 5.0 Hz	7.45 ddd	7.94 ddd J_{CD} = 7.60 Hz	7.57 ddd J_{DC} = 7.60 Hz
$Zn^{2+}/9$ = 0.5	7.77 ddd J_{AB} = 5.40 Hz	7.40 ddd	8.25 ddd J_{CD} = 7.60 Hz	8.54 ddd J_{DC} = 7.60 Hz
d-9	—	7.45 dd J_{BC} = 7.6 Hz J_{BD} = 1.0 Hz	7.92 dd J_{CD} = 7.6 Hz J_{CD} = J_{CB} = 7.6 Hz	7.54 dd J_{DC} = 7.6 Hz J_{DB} = 1.0 Hz
Zn^{2+}/d-9 = 0.5	—	7.43 dd J_{BC} = 7.6 Hz J_{BD} = 1.0 Hz	8.26 dd J_{CD} = J_{CB} = 7.6 Hz	8.56 dd J_{DC} = 7.6 Hz J_{DB} = 1.0 Hz

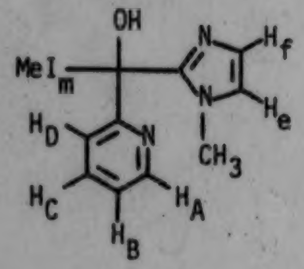
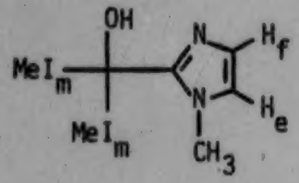
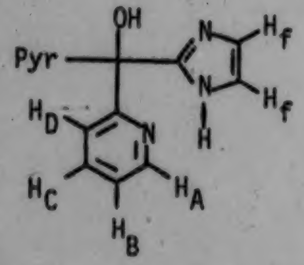
.......continued

Table VI (continued)

Ligand	δ and J value						
	CH_3	H_f	H_e	H_A	H_B	H_C	H_D
10 	3.47 s	7.24 s	6.93 s	8.57 dm	7.46 m	7.95 dd	7.51 dm
Zn^{2+}/10 = 0.5	4.21 s	6.44 s 6.62 s	7.10 s	8.04 dm	7.42 m	8.22 td	8.46 dm
Zn^{2+}/10 = 15	4.08 s	7.18 d (or H_e)	7.12 d (or H_f)	8.78 dm	7.63 ddd	8.30 overlapping	8.08
d-10	3.48 s	7.24 s (br)	6.94 s (br)	—	7.52 dd J_{BC} = 5.2 Hz	7.96 dd $J_{CD} = J_{CB}$ = 5.2 Hz	7.52 dd
Zn^{2+}/d-10 = 0.5	4.27 s	6.42 s 6.60 s	7.08 s (br)	—	7.40 d (br) J_{BC} = 5.2 Hz	8.20 dd $J_{CD} = J_{CB}$ = 5.2 Hz	8.45 d (br)
Zn^{2+}/d-10 = 15	4.08 s	7.19	7.14 s		7.65 dd J_{BC} = 4.8 Hz J_{BD} = 1.6 Hz	8.30 overlapping	8.08

.......continued

Table VI (continued)

Ligand	δ value						
	CH_3	H_f	H_e	H_A	H_B	H_C	H_D
11 	3.52 s	7.31 d	7.01 d	8.71 dm	7.62 dd	8.07 dd	7.40 dm
12 	3.43 s	7.24 d	7.00 d	—	—	—	—
13 	—	7.18 s	—	8.56 ddd	7.46 ddd	7.94 ddd	7.55 ddd

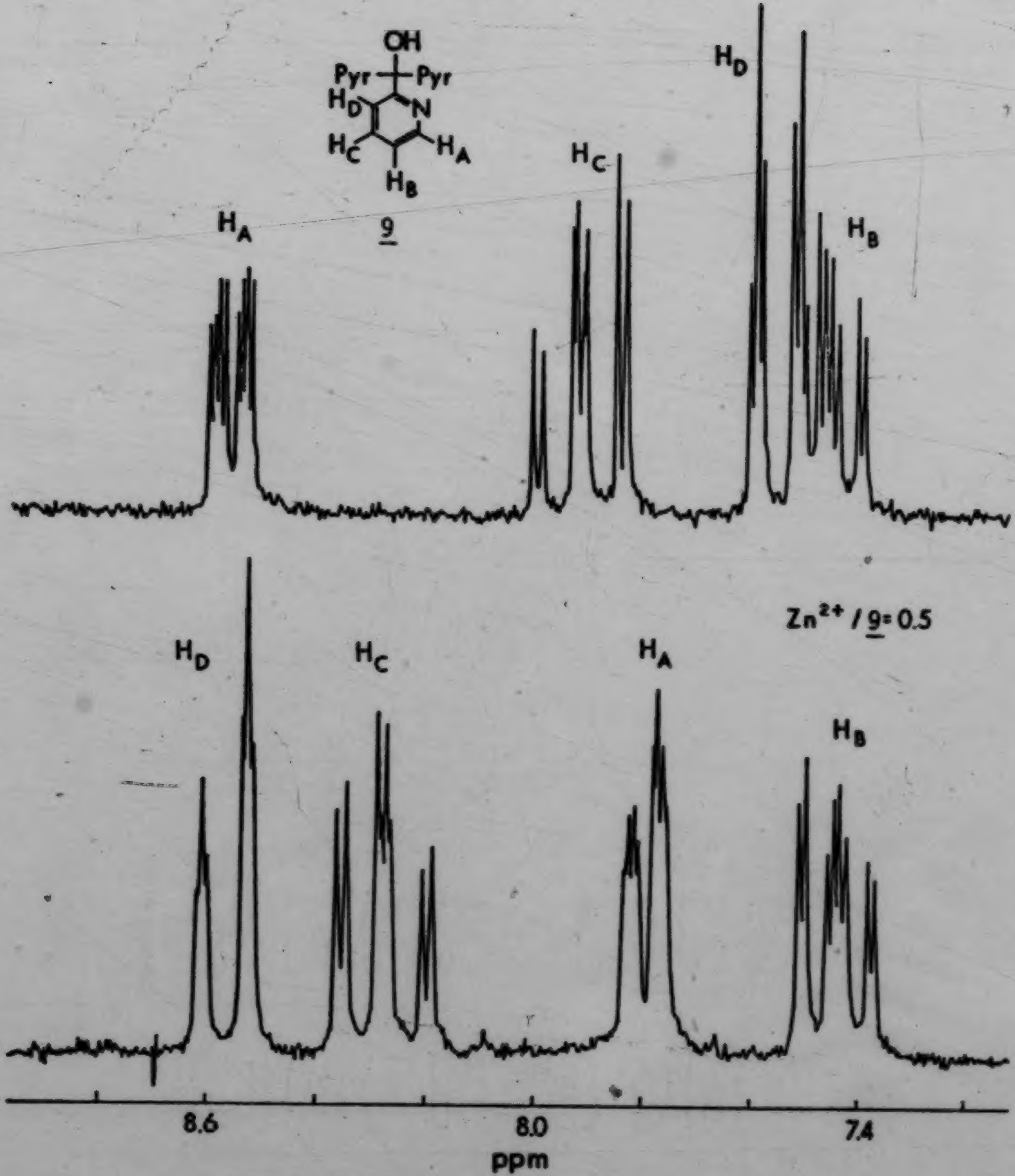


Fig. 3. The ^{1}H-nmr spectra of 9 and its 2:1 Zn^{2+} complex in D_2O. See Table VI.

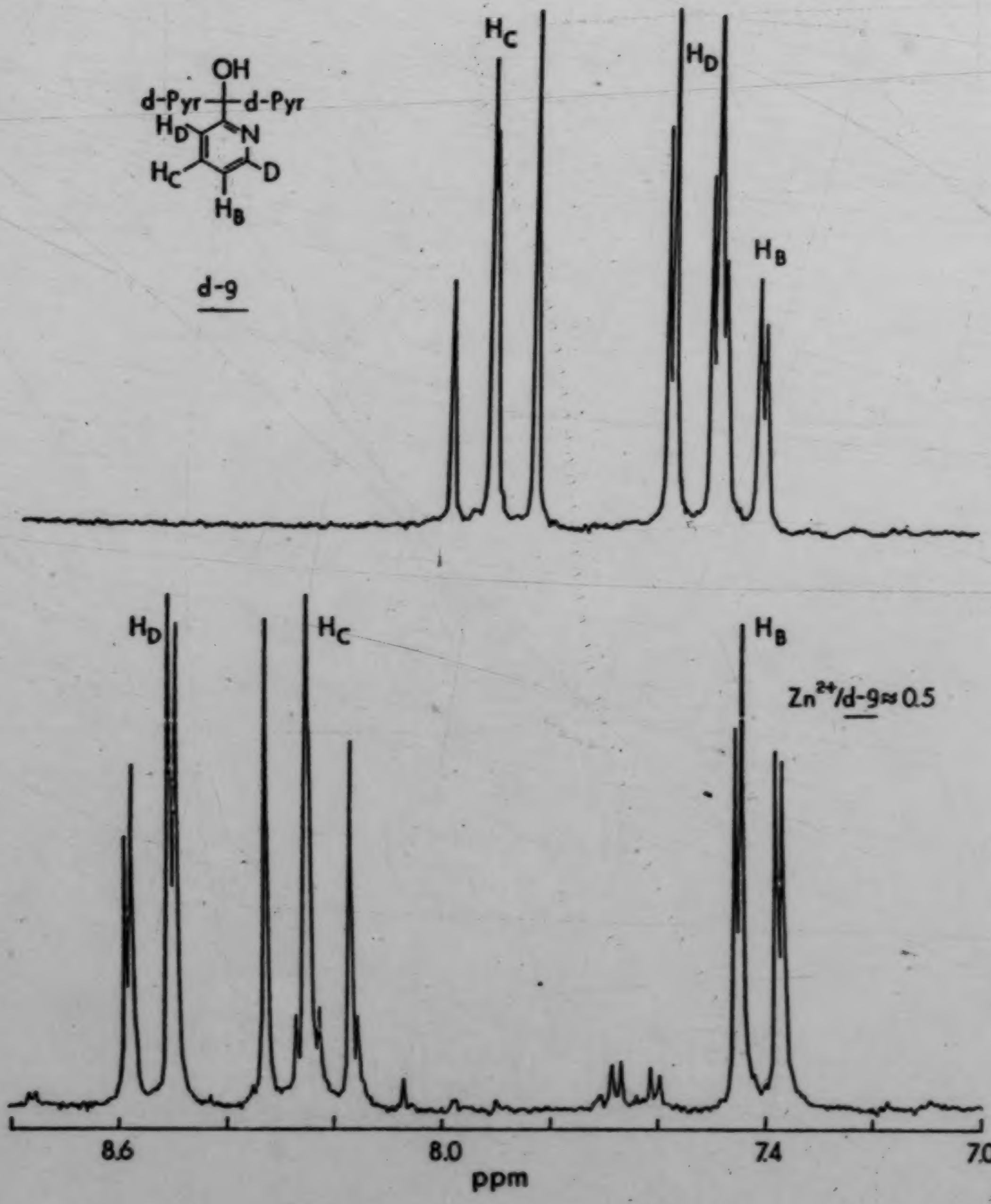


Fig. 4. The ^{1}H-nmr spectra of d-9 and its 2:1 Zn^{2+} complex in D_2O. See Table VI.

in the 2:1 complex lies very close to and above the plane of a pyridine group of a second ligand**, and is therefore shielded by the latter's ring current. Unambiguous confirmation of these assignments is provided by the spectrum of the analogous 2:1 complex of d-9 in Fig. 4. These observations are important when considering the spectra for 10 and its Zn^{++} complexes illustrated in Fig. 5. The middle spectrum again shows downfield shifts for H_D and H_C and an upfield shift for H_A; the position of H_B remains roughly unchanged. A sizeable downfield shift of 0.74 ppm is observed for the imidazole $N\text{-}CH_3$, as well as some small signals attributable to some residual uncomplexed 10. Increasing $[Zn^{++}]$ causes a new set of resonances to appear at the expense of those from 10 and its 2:1 complex, until a symmetric pattern is obtained when the $Zn^{++}/10$ ratio is 15. We believe that this final spectrum is that of the 1:1 complex of 10 and note that the pyridine H_A has shifted downfield once it is removed from the proximity with the second ligand. The spectral parameters for d-10 confirm the assignments (Table VI and Fig. 6).

From the above, one can reasonably conclude that these molecules exist as symmetrically bound complexes in solution, and that both 2:1 and 1:1 complexes exist

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** This same effect has been noted before in the nmr spectrum of $[Fe^{II}(Bipyr)_3]Cl_2$[61].

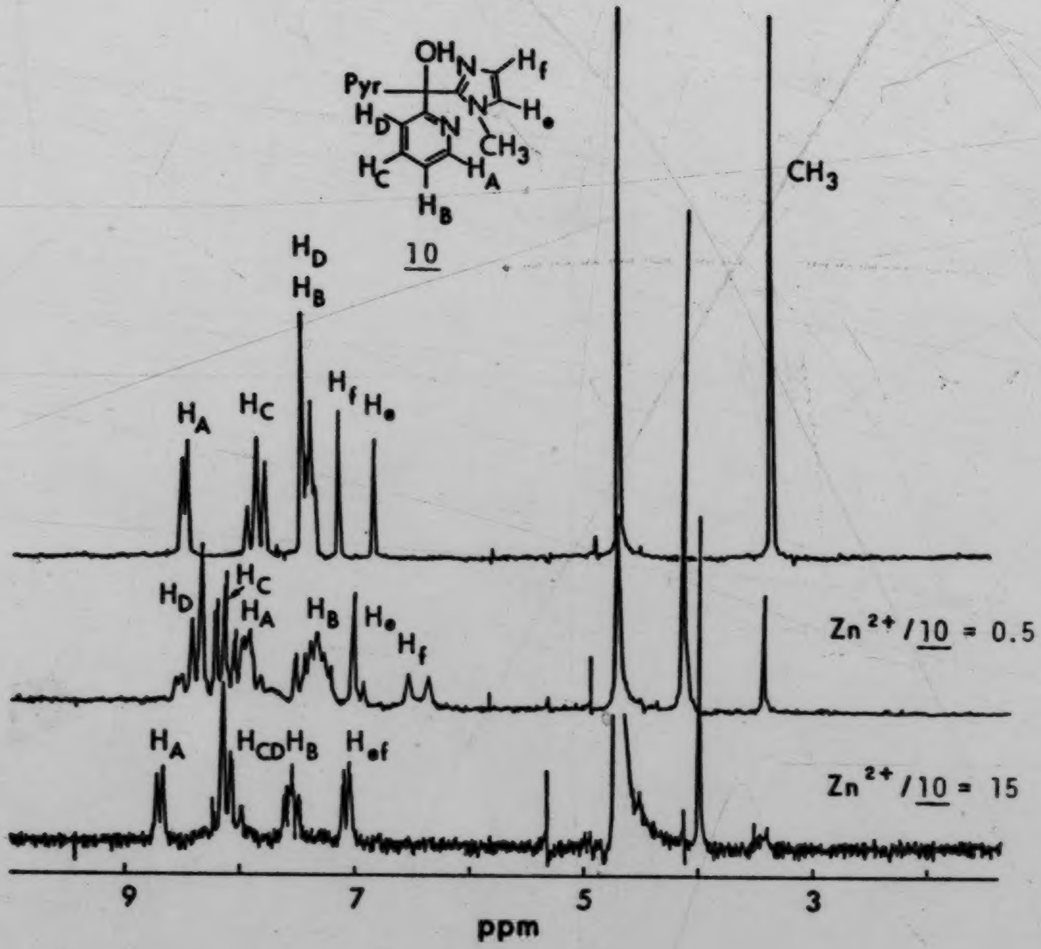


Fig. 5. The ^{1}H-nmr spectra of 10 as a function of increasing [Zn^{2+}]. See text and Table VI for assignments. The resonance at δ 4.8 is attributable to HOD.

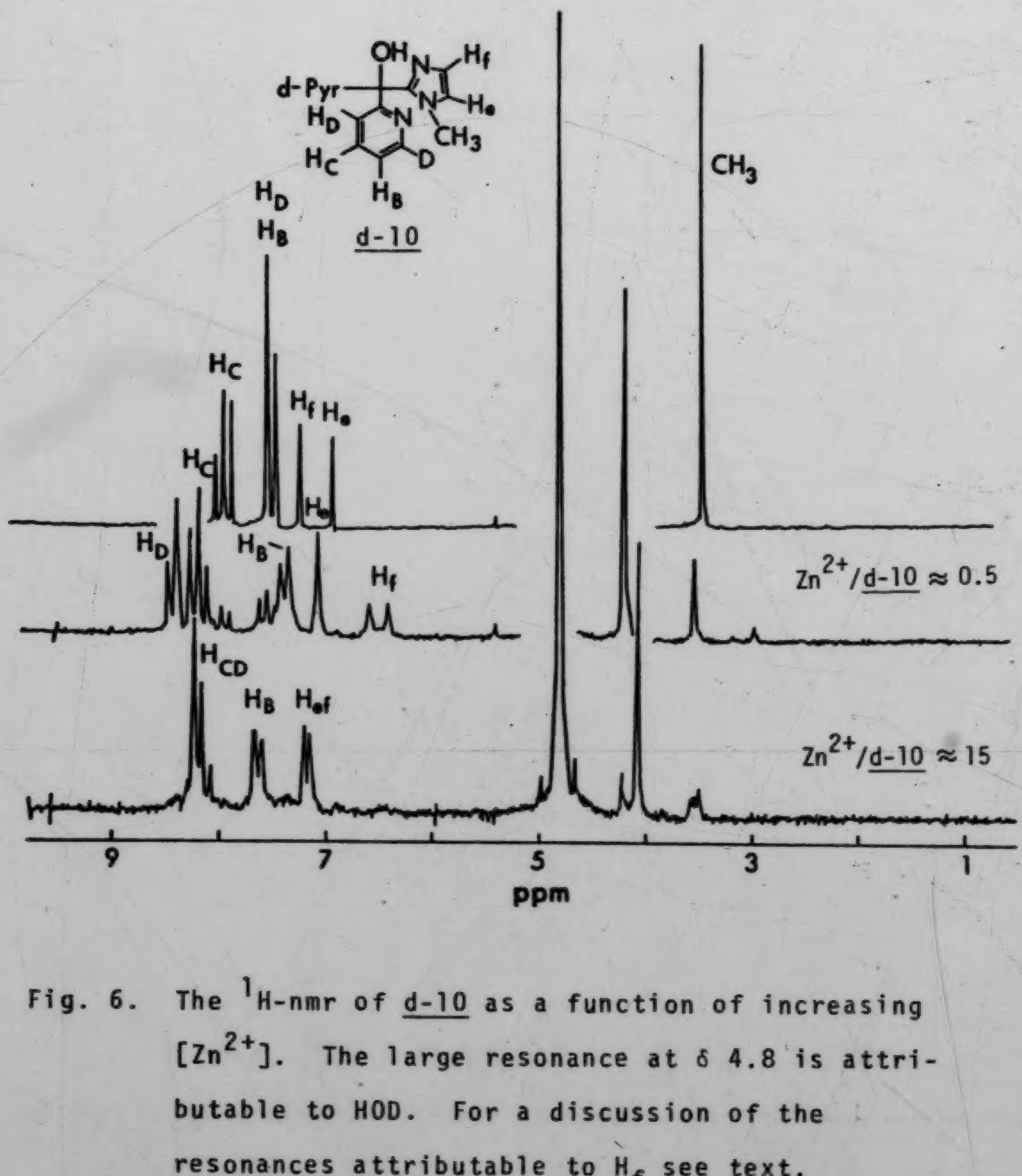


Fig. 6. The ^{1}H-nmr of d-10 as a function of increasing [Zn^{2+}]. The large resonance at δ 4.8 is attributable to HOD. For a discussion of the resonances attributable to H_f see text.

depending on the ligand:Zn^{++} ratio. It is noteworthy that for the equilibrium described in eq. 19, the rate of interchanging the species is slow on the nmr time-

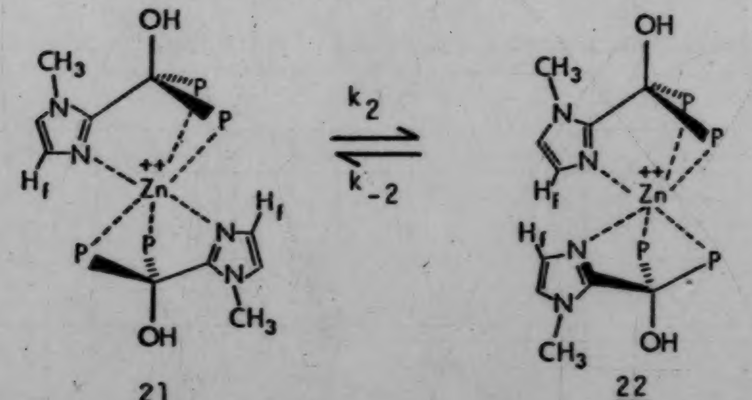
$$2L + Zn^{++} \rightleftharpoons \underset{\text{(1:1 complex)}}{L + L{:}Zn^{++}} \rightleftharpoons \underset{\text{(2:1 complex)}}{L{:}Zn^{++}{:}L} \qquad (19)$$

scale since separate resonances are observed for each species.

Analogous experiments performed with ligands 16 and 17 showed very broad and ill-defined nmr spectra, indicating unsymmetrical binding and/or fast exchange between complexed and non-complexed ligand.

Rotation within the 2:1 complex of 10 -

From the middle spectra in Fig. 5 and Fig. 6 it can be seen that the imidazole hydrogens, H_e and H_f, appear as a singlet at δ 7.10 and a pair of fortuitously equal intensity broadened singlets at δ 6.44 and δ 6.62 respectively. From equation 20 in the symmetrically bound 2:1 complex H_f of one imidazole can be positioned



(20)

21 22

between two pyridines of the second ligand (21) or between an imidazole and a pyridine of the second ligand (22). This should give rise to two different shieldings for H_f, the H_f peak from 22 twice as intense as the one from 21. The fact that both peaks appear of equal intensity can be explained if for example 21 is fortuitously a little more stable than 22, the equilibrium constant for the process in equation 20 being $K_{eq} = k_2/k_{-2} = 1$. Variable temperature ^{1}H-nmr studies on this sample showed that at temperatures above 320 K, the latter two peaks coalesce into a singlet centered at δ 6.52, experimentally midpoint between the H_f values shown in Table VI. At 275 K, two approximately equal intensity imidazole N-CH_3 resonances separated by 2 Hz were observed, in agreement with the described equilibrium mixture of 21 and 22.

The rate of site exchange (k_2, equation 21) and the activation energy ($\Delta F^{\ddagger}$, equation 22) for the process could be calculated assuming equal populations in both sides of the equilibrium[62]. From the experimental $\Delta\nu_{H_f}$ = 20 Hz (at 100 MHz) and a coalescence temperature of 320 K

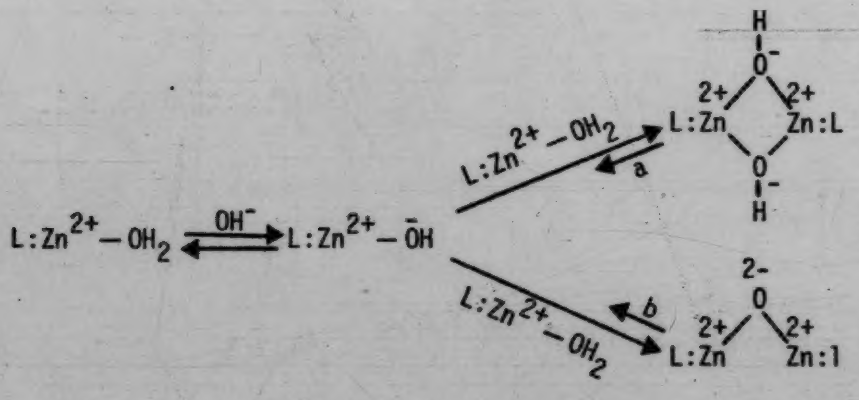
$$k_2 = 1/2\,\tau = 1/[2(0.225/\Delta\nu)] = 44\ \mathrm{sec}^{-1} \qquad (21)^{62}$$

$$\Delta F^{\ddagger} = 2.303\,RT(10.319 + \log T - \log k_2) = 16.4\ \mathrm{kcal \cdot mol^{-1}} \qquad (22)$$

5. Zn^{++} COMPLEX TITRATION

Of particular importance to the activity of C.A., and therefore to any system designed to mimic C.A., is the activity related ionization of a group with a pK_a ~ 7 (see Introduction). Among other candidates, $Zn\text{-}OH_2$ has been theorized to have such a low pK_a in the enzyme[1a,1j, 20,24,26,27] and it has been demonstrated in some model systems[31] that the pK_a of the zinc-bound water may approach 7 if the coordination number of zinc in the complex is 4 or 5.

On the other hand, ionization of Zn-bound imidazole has been proposed to account for the low pK_a[28,40] with some experimental justification in simple complexes[40]. In order to cast some light on the above question, ligands 10 and 13, differing only by N-methylation of the imidazole were complexed to an equivalent amount of Zn^{++} and titrated with NaOH. In both cases titration of the free bases (0.025 mmol) in a solution containing 0.1095 mmol HNO_3 showed complete release of all added protons after pK_{a1} had been passed, and that this process was completely reversible. When bound to equimolar Zn^{++}, 13 showed complete release of all added H^+ by pH ~ 3.5 indicating that metal binding was complete by that point, and the additional release of 0.025 mmol H^+ (1.0 equiv. based on 13:Zn^{++}) upon further titration, suggesting Zn^{++}-OH_2 or Zn^{++}-ImH ionization, with an apparent pK_a of 6.98. Ultraviolet spectra of the solution on either side of the apparent pK_a showed no substantial changes and the solution remained clear until at least pH 10, indicating that free [Zn^{++}] was very low. Analogous titration of 10 in the presence of Zn^{++} curiously showed the release of only 0.8 equiv. of H^+ (based on 10:Zn^{++}) at the apparent pK_a of 6.50 and not the expected 1.0 equiv. The difference between the observed and the expected base consumption (in this system) may be associated with the presence of 2:1 complex. Again, UV spectra of the solutions above and below the apparent pK_a showed minimal differences. On the surface, the data appear to be most consistent with ionization of a Zn-OH_2, however this is tempered by the fact that neither titration proved to be reversible. A speculative explanation for the irreversibility of the titrations might be dimer formation as in path a or b of equation 23, although one would think that these



(23)

"dimers" would revert back to L-Zn^{++}-OH_2 quickly in acid solution. The characterization of these dimers was attempted by isolating a complex from basic solutions containing ligand and Zn^{++}. However, the ^{1}H-nmr spectrum, microanalysis and molecular weight determination gave inconclusive results. While some precedence for the existence of oxy and hydroxy bridged Zn^{++} dimers is available[63] such bridged species are more commonly found with ions like Co(II), Cu(II), Ni(II), and Fe(III)[64].

Ligands 14-17 were investigated analogously. It can be seen from space filling models that the progressively bulkier substituents at the 4,5-positions should prevent 2:1 (L:Zn^{++}:L) binding, and reduce the possibility of bridged dimers since there should be severe buttressing between the *iso*propyl groups on adjacent ligands. Titration of Zn^{++}:14 showed the consumption of 1.0 equiv. of OH^- at about pH 7, but this was tied to the reversible formation of an intensely blue-colored solution which indicates the ligand itself suffers some modification. Ligand 15, even at acid pH, when complexed with Zn^{++} gave a bright pink solution, consistent with transformation to 19 according to equation 16.

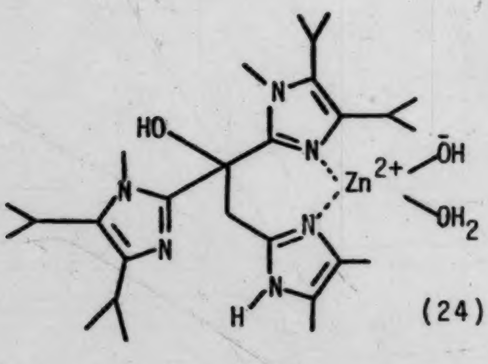
Ligand 16 on the other hand showed no propensity to form colored solutions (again supporting dehydration of 14 and 15) and was therefore titrated (for reasons of solubility 16 required a titration medium of 80%

aq. dioxane) in the presence of equimolar Zn^{++}. In this case, curiously at "pH" 7 two equivalents of OH^- were consumed and the process was reversible. Control experiments under the same conditions but in the absence of 16 showed again the consumption of two equivalents of OH^- at a "pH" of 7, which is clearly tied to the formation of $Zn(OH)_2 \cdot XH_2O$, but the latter species appears quite soluble in the medium. Thus it would appear that because Zn^{++} binds to 16 only weakly ($pK_{Zn^{++}} \approx 4$, Table V) at elevated "pH" OH^- simply sequesters the metal away from the ligand.

Ligand 17 was designed to prevent 2:1 ($L:Zn^{++}:L$) binding, oxy- or hydroxy-bridged dimers, and the deleterious dehydration in the presence of Zn^{++} and OH^-. One imidazole N-H was required to provide reasonable metal binding. Titration of 17 in the presence of equimolar Zn^{++} showed the reversible consumption of 1 equiv. OH^- with an apparent "pK_a" of ~ 6.5 in 76% ethanol/water and of ~ 5.8 in 60% aq. dioxane. In the absence of 17, Zn^{++} under the same conditions showed precipitation of $Zn(OH)_2$ and the consumption of 2 equiv. OH^- at around "pH" 7. Interaction of 17 with Zn^{++} is strong enough to hold the latter in solution, and the consumption of OH^- is consistent with $Zn^{++}:OH_2$ or $Zn^{++}:ImH$ titration (although the "pK_a" seems too low) or more likely with OH^- displacement of one of the weakly bound $N-CH_3$

imidazoles as in equation 24.

$$\underline{17}:Zn^{2+}OH_2 + OH^- \rightleftharpoons$$



(24)

6. Co(II) COMPLEX TITRATION AND UV VISIBLE SPECTRA

The fact that activation of apo-C.A. can be achieved with Co(II)[7] provides the possibility of using the spectroscopic properties of cobalt as a probe for the active site geometry of C.A. Zn(II) can not give the same kind of information because it has a complete set of 3d electrons.

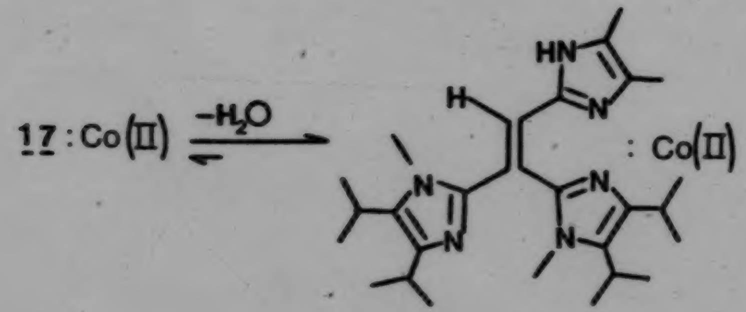
The Co(II) ion has a d^7 configuration, and it forms preferentially octahedral complexes, although four and five coordination are not uncommon[7b,65]. The d-d absorption spectra of Co(II) are so characteristic that the geometry of a complex can be reasonably well predicted from its spectrum[65b,65c]. Generally octahedral Co(II) is pink (λ_{max} ~ 500 nm, ε~ 10 M^{-1} cm^{-1}), and tetrahedral is blue or violet ($\lambda_{max} \gtrsim$ 575 nm, ε~ 200-1000 M^{-1} cm^{-1}). High-spin pentacoordinated Co(II) complexes have absorption bands usually between 500 and

700 nm with intensities intermediate between those of tetrahedral and octahedral species[10,65c].

Again, ligands 10 and 13 were compared as their Co(II) complexes. Titration of equimolar amounts of 10 and Co(II), and 13 and Co(II) showed titration of a group in each one of the complexes, with respective pK_a's of 8.75 and 8.04. Ultraviolet spectra of each above and below the apparent pK_a did not show evidence for tetrahedral or five coordinated Co(II). Hence in these systems what appears to be simple $Co-OH_2$ titration is not sufficient to enforce a tetrahedral geometry.

Ligands 9, 11, and 12 showed absence of any tetrahedral or five coordinated Co(II) bands.

The complex 17:Co(II) upon titration with NaOH in 76% aq. ethanol and in the absence of air developed a violet color (λ_{max} = 550 nm, ε = 60 assuming complete 1:1 binding) attributable to four or five coordination with an apparent $pK_a \sim 7$. However, no conclusion could be drawn from this experiment, since the violet color formation proved not to be reversible upon reacidification with perchloric or hydrochloric acid. This irreversibility is not likely due to Co(III) formation because the experiments were performed in the absence of oxygen, but probably to ligand decomposition (dehydration?) as in equation 25.



(25)

7. CATALYTIC STUDIES

Ligands 9 and 17 were tested for catalytic activity towards PNPA hydrolysis at several pH's around neutrality, in the presence of several Zn^{++} concentrations. For both ligands no catalytic rate enhancement was detected. Ligand 17 was also tested for acetaldehyde hydration without success. Finally 17 was tested for CO_2 hydration in 76% aq. ethanol. The results are summarized in Table VII.

The first noticeable thing is the high values for k_{obs} compared to literature values[66] obtained for CO_2 hydration in aqueous solutions under similar buffer and ionic strength conditions. This is in agreement with Caplow's[67] observation that the bicarbonate dehydration is accelerated 43 times relative to water in the presence of 71.5% dioxan. Probably the transition state[66c] does not have much charge separation (equation 26) and is

TABLE VII

CO_2 hydration in 76% ethanol:H_2O at 25°.

Reaction conditions		k_{obs} (sec^{-1})	k_{cat}[d] (M^{-1} sec^{-1})
Spontaneous	(pH 7.50)	0.175±0.015	—
Spontaneous	(pH 6.50)	1.110±0.050	—
17[b]	(pH 7.50)	0.180±0.005	e
17 + Zn^{++}	(pH 7.50)	0.284±0.012	109±27
17 + Zn^{++}	(pH 6.50)	1.520±0.025	410±75

[a] Kinetics measured in 0.05 M HEPES buffer. Ionic strength was kept constant at 0.2 M by $NaClO_4$ addition. pH values are those directly read from electrode immersed in solution.

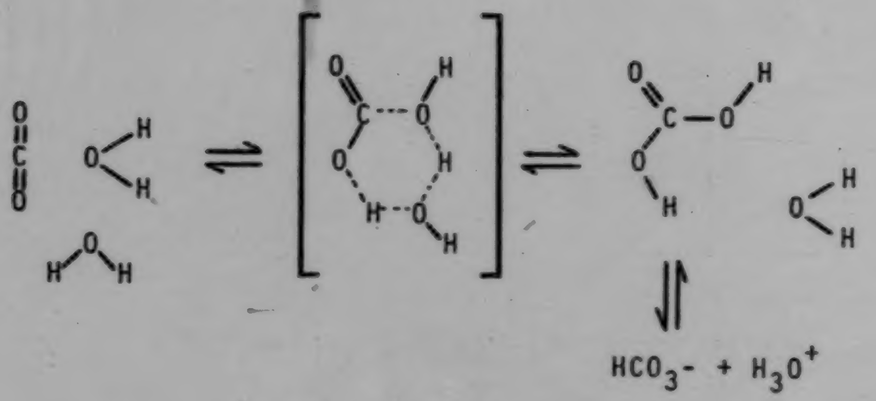
[b] [17] = 10^{-3} M

[c] [17] = [$Zn(ClO_4)_2$] = 10^{-3} M

[d] $k_{cat} = (k_{obs} - k_{spont})/[cat]$

[e] Negligible within experimental error.

stabilized by decreasing the polarity of the medium.

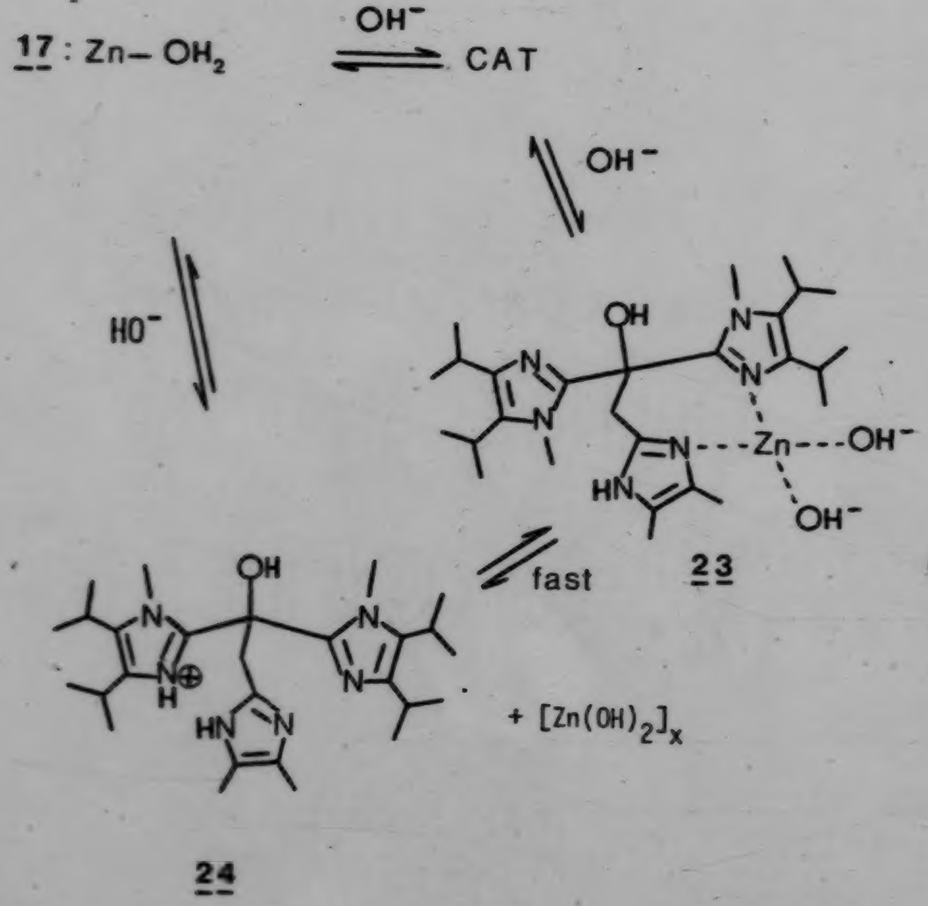


(26)

The second and most important observation is that k_{cat} diminishes with increasing pH. Although measurements performed only at two different pH values are not enough to define the pH dependence of the phenomenon measured, it is clear that k_{cat} at pH 7.50 is smaller than at pH 6.50. In other words, the concentration of the catalytically active species ([CAT]) is decreased as pH increases.

Assuming that the catalytically active species is a *tris*-imidazole-coordinated zinc complex, the following equilibrium (equation 27) can be proposed, which is also consistent with the base titration of the complex.

CAT could be a zinc bound hydroxide species. At pH 6.5 the concentration of CAT would be enough to produce the observed increase of k_{obs} of 0.410 sec^{-1}. When more base is added to the system to increase the pH to 7.50, the concentration of CAT (probably through 23)


17 : Zn– OH₂
OH⁻
CAT
OH⁻
HO⁻
OH
N
N
HN
Zn
OH⁻
OH⁻
23
fast
OH
N
N⊕
H
HN
N
+ [Zn(OH)₂]ₓ
24

(27)

is decreased. Zinc hydroxide could then precipitate or agglomerate irreversibly and the ligand could protonate at pH 7.5 since pK_{a_1} for 17 is 8.2. The base consumption in going from 17:Zn-OH_2 to 24 would still be one mole of base per mole of complex.

8. CONCLUSION

Although for various reasons each of ligands 9-17 has some serious deficiency in terms of their ability to be considered as "models" for the metal binding site of C.A., several important observations have been made which cast light on the minimum features such models must possess.

1. N-CH_3 imidazole ligands bind metal rather poorly when compared with their N-H analogues. One clearly requires large $pK_{M^{++}}$ values for a potential model for C.A. so that the metal will not be sequestered away from the ligand at elevated pH.
2. The small ligands 9-12 from nmr studies in D_2O solution can exist as 2:1 or 1:1 metal complexes, the latter being favored at high metal concentrations.
3. Deleterious 2:1 ($L:M^{++}:L$) binding can be overcome by placing large alkyl groups at the 4,5-imidazole positions. However such substitution in the *tris*-imidazole carbinol series appears to lead to facile dehydration to produce highly colored fulvene-like materials. Such dehydration can be overcome by removing the carbinol OH group, or methylating the imidazole nitrogen, however the former substitution of OH by H produces an extremely easily air-oxidized methane, and the latter N-methylation

leads to rather poor metal binding ability.

One possible reason for the dehydration of the N-H ligands and poor binding of the N-CH_3 analogues might be strain forces involved in the formation of the metal complex. The fact that ketone 16b can be obtained by simply heating of carbinol 16 (SCHEME XIII) indicates that 16 itself might be a strained molecule and decomposition relieves this strain. Binding to metal strains even more the ligand molecule. Figure 7 shows the approximate geometrical arrangement for Zn^{++} binding of one imidazole of a carbinol ligand. Bond lengths (OH)C-C_2 = 1.47 Å, C_2-N_1 = 1.33 Å, and N_1-Zn = 2.05 Å, and angles N_3-C_2-N_1 = 109°, and

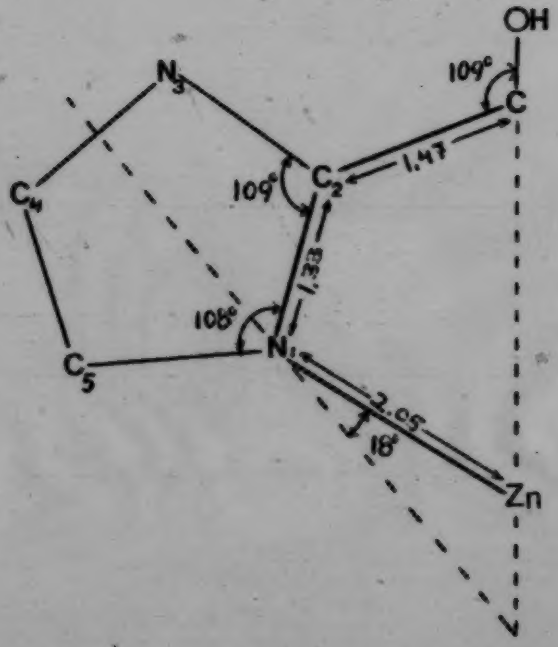


Fig. 7. Geometrical arrangement for Zn^{++} binding of one imidazole of a carbinol ligand.

C_2-N_1-C_5 = 108° were assumed to be the same as the analogous ones in the complex 16b:$ZnBr_2$ (APPENDIX III). The angle HO-C-C_2 was assumed to have the tetrahedral value of ~109°. It can be seen that C-C_2-N_1-Zn form half of a very distorted hexagon. Assuming that the optimum direction for imidazole binding is the one that bisects the C_2-N_1-C_5 angle, as represented by the dotted line in Fig. 7, the deviation from this angle when the *tris*-imidazolyl-carbinol ligands bind Zn^{++} is quite large (~18°). In order to minimize this deviation, the molecule has to increase the OH-C-C_2 angle, thus increasing its total energy, and as a consequence its susceptibility to chemical modification.

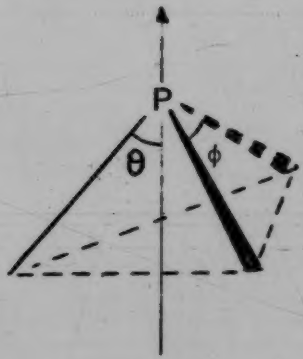
From the above, the next step in the construction of a "model" for the active site of C.A. was the design of a series of ligands lacking the possibility for dehydration, and making metal binding more favorable, without the need for a large distortion of the molecule (i.e.: the phosphine analogues of the carbinols).

D. PHOSPHINES

As indicated in the previous section the *tris*-imidazolyl-phosphines were synthesized in order to avoid some problems presented by the carbinol analogues. A similar geometrical analysis of the mode of binding to

Zn^{++} can be done.

The geometry of phosphines is that of a trigonal pyramid[69,70] (Fig. 8). The angle each bond makes



$$\cos\theta = \left[1 - \frac{4}{3}\sin^2\left(\frac{\phi}{2}\right)\right]^{1/2} \qquad (28)$$

Fig. 8. Geometry of Phosphines

with the principal axis (θ) is related to the bond angle (ϕ) by equation 28.[70] The bond angle ϕ is typically around 100° (ref. 70). With this value and using equation 28 a value of 62° for θ is obtained. The average value for P-C bonds in trisubstituted phosphines is 1.8 Å[70]. With this data and the N-Zn bond distance of 2.05 Å (APPENDIX III), the approximate geometrical arrangement for Zn^{++} binding of a *tris*-imidazolylphosphine can be drawn (Fig. 9).

It is observed that the deviation of the actual N-Zn binding direction from the optimum one (dotted line) is smaller than in the carbinol case (~13°). This indicates, however, that these phosphines do not afford yet the optimum geometry for Zn^{++} binding.

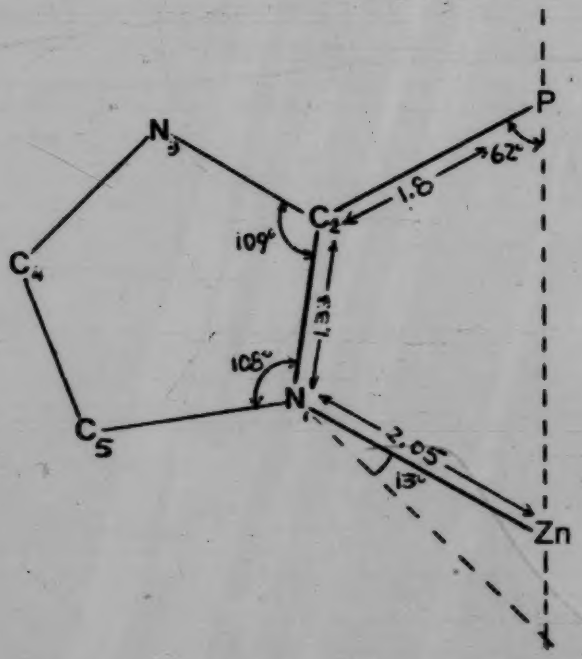


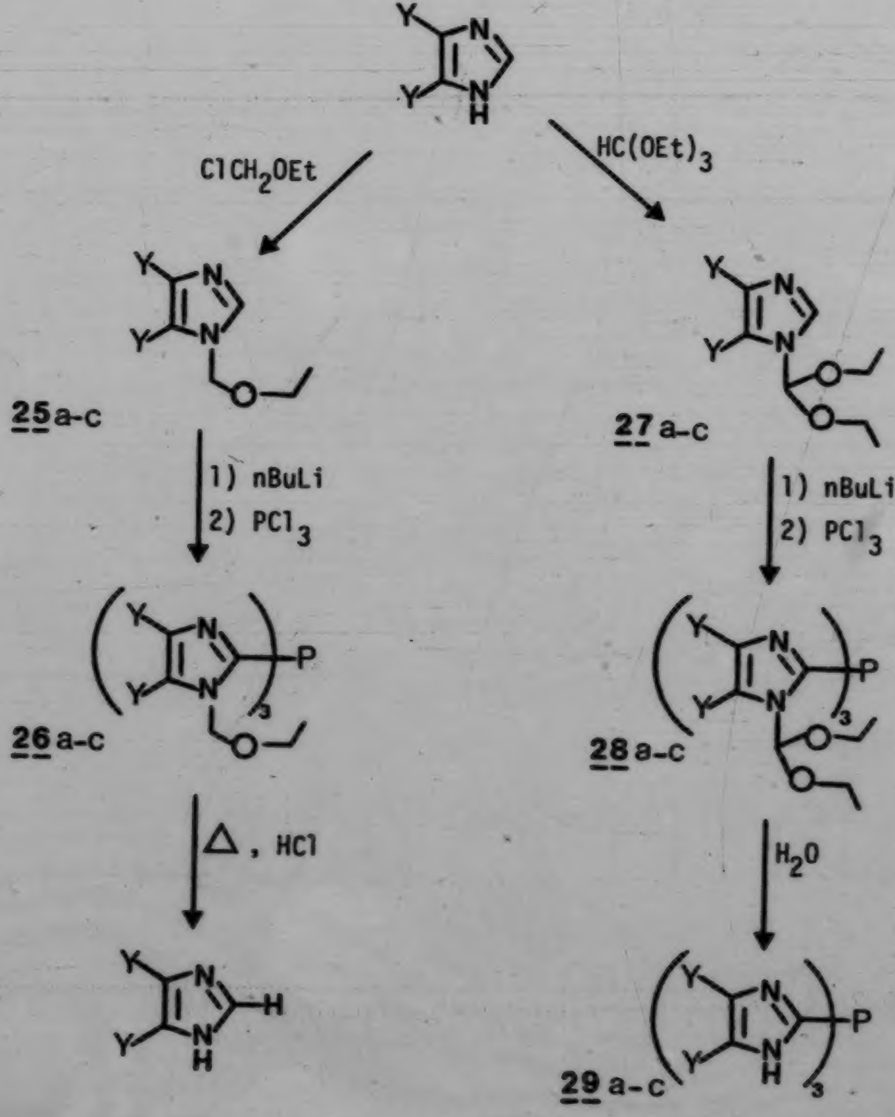
Fig. 9. Approximate geometrical arrangement for Zn^{++} binding of a *tris*-imidazolyl-phosphine.

1. SYNTHESIS

Nucleophilic displacement on phosphorous halides by organometallic reagents is a very facile process leading to tertiary phosphines[71].

The synthesis of phosphines 29(a-c) (Scheme XIV) was attempted first by reaction of lithiated 25(a-c) with phosphorous trichloride. The phosphines 26(a-c) were obtained, but the strong acid conditions needed to deprotect the imidazoles[44] led to P-C bond cleavage.

SCHEME XIV


ClCH$_2$OEt
HC(OEt)$_3$
25a-c
27a-c
1) nBuLi
2) PCl$_3$
26a-c
28a-c
Δ, HCl
H$_2$O
29a-c

a Y = H
b Y = Me
c Y = *i*-Pr

Instead, the *bis*-ethoxy methyl was used as a protecting group[72]. The N-protected imidazoles 27(a-c) were lithiated and reacted with phosphorous trichloride to give the phosphines 28(a-c) which were not isolated but successfully deprotected under neutral conditions to afford compounds 29(a-c).

2. H^+ AND M^{++} BINDING CONSTANTS

Ionization and metal binding constants were determined in the same way as for the carbinol compounds. Ionization constants are listed in Table VIII. Due to solubility reasons the experiments had to be performed in media of different ethanolic content, therefore the values for the three ligands are not directly comparable. For example, phosphine 29c appears to be substantially less basic than 29b, but that might likely be a solvent effect, the more polar environment (less ethanol content) giving a higher apparent pK_a.

Zn^{++} and Co^{++} binding constants to ligand 29c were determined in the same medium used for pK_a's determination, and found to be 5.90 ± 0.05 and 3.70 ± 0.20 respectively. The much better ability to bind Zn^{++} compared to Co^{++} is probably due to tetrahedral binding[58]. However, these binding constants are very small if one compares them with the affinities for Zn^{++} and Co^{++} of

TABLE VIII

Ionization constants (pK_a's) for ligands 29(a-c)[a].

Ligand	pK_{a1}	pK_{a2}	pK_{a3}	EtOH/H_2O ratio[b]
29a[c]	6.79	6.04	2.54	0.67
29b[d]	7.58	6.51	2.44	0.20
29c[e]	6.60	4.35	2.80	4.00

[a] ± 0.05 unit precision.

[b] at the beginning of the titration.

[c] 4 ml 0.25 M KNO_3 in 1:1 EtOH/H_2O, 1 ml 0.025 M L in 0.1051 M HNO_3.

[d] 4 ml 0.25 M KNO_3, 1 ml 0.025 M L in EtOH, 1 ml 0.1051 M HNO_3.

[e] 3 ml EtOH, 75 mg KNO_3, 1 ml 0.1051 M HNO_3, 1 ml 0.025 M L in EtOH.

apo-carbonic anhydrase. Perhaps this results from the non-optimized geometry for metal binding of this phosphine.

3. NUCLEAR MAGNETIC RESONANCE STUDY OF 29c AND Zn^{++} BINDING

^1H- nmr spectra of roughly 0.026 M 29c in d_4-

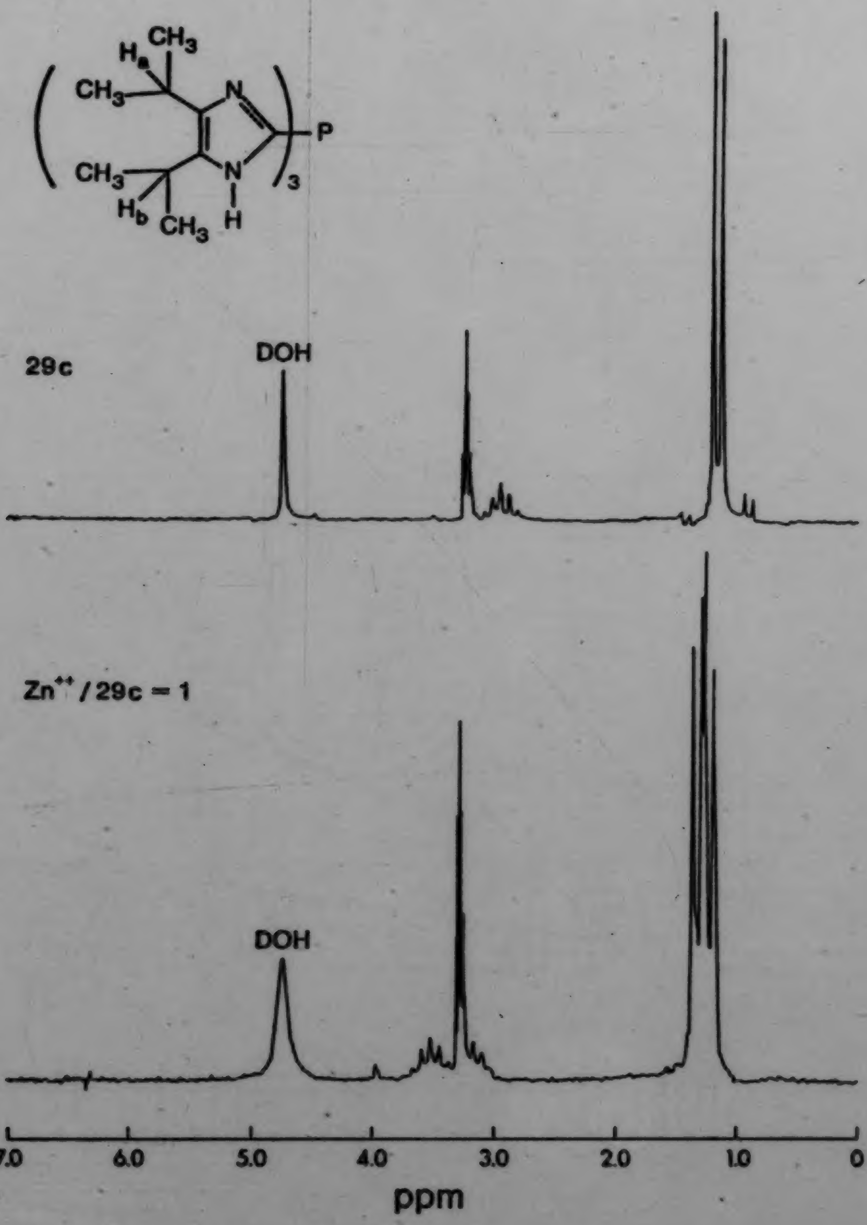


Fig. 10. The ^{1}H-nmr spectra of 29c and its 1:1 Zn^{++} complex in D_2O-CD_3OD. See Table IX.

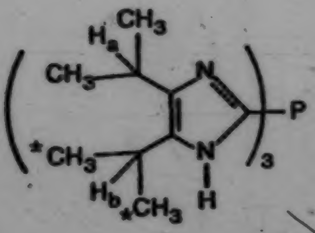
methanol:D_2O were recorded as a function of increasing $[Zn^{++}]$. As it can be seen in Fig. 10, when the 29c/Zn^{++} ratio was 1, a well-defined symmetrical 1:1 complex was observed. Chemical shifts and coupling constants for the ligand and its Zn^{++} complex are given in Table IX. Further addition of Zn^{++} did not change the spectrum and 2:1 complex was not observed.

For the equilibrium

$$\underline{29c} + Zn^{++} \rightleftharpoons \underline{29c}:Zn^{++} \qquad (29)$$

TABLE IX

Chemical shift values for ligand 29c and its Zn^{++} complex determined in $CD_3OD:D_2O$ solution.

		H_a	H_b	CH_3	$^*CH_3$
	29c	3.00 sept J = 7 Hz		1.22 d J = 7 Hz	
$[Zn^{++}]/[\underline{29c}] = 1$		3.53 m	3.17 m	1.34 d J = 7 Hz	1.22 d J = 7 Hz

the rate of interchanging the species is slow on the nmr timescale since separate resonances are observed for each species. Also, the fact that two distinct resonances for the *iso*propyls are observed indicates that the molecule is indeed bound tridentate as expected.

4. RATE OF Zn^{++} BINDING BY 29c

There is evidence that the binding of metal ions by apo-carbonic anhydrase is different in important respects from the binding of the same ions by small ligands.

For example, the second order rate constants for the reaction of Zn^{++} with small ligands are in the range 10^7-10^8 M^{-1} sec^{-1}, with little dependence on the nature of the ligand[73]. Holyer et al[74] reported lower values, close to 10^6 M^{-1} sec^{-1} at 25°, for the reaction of Zn^{++} with 1,10-phenanthroline, 2,2'-bipyridine, and 2,2',2"-terpyridine. The reaction of Zn^{++} with apo-C.A. is characterized by rates which are two orders of magnitude slower than observed with these polydentate ligands[75].

The activation parameters for the reaction of Zn^{++} with the apoenzyme are also very different compared to the reaction with the ligands studied by Holyer et al[74]. Chelate formation with small ligands is characterized by a low energy of activation of 7-8 kcal $mole^{-1}$ and a small negative entropy of activation of -4 to -8 cal deg^{-1} $mole^{-1}$, whereas the protein reaction has an unusually large energy of activation, 21 kcal $mole^{-1}$, which is partially compensated by a fairly large positive entropy of activation amounting to 27-29 cal deg^{-1}

$mole^{-1}$[75]. Since the Zn^{++} is bound to the protein at the bottom of a cleft, the large positive entropy of activation has been attributed[75] to liberation and disorder of bound water molecules from the Zn^{++} and/or the protein cavity.

In order to compare it with the enzyme, the rate of Zn^{++} binding by 29c was studied in 76% ethanol:water (again the organic medium was needed for solubility reasons). The results are summarized in Table X.

TABLE X

Rates of Zn^{++} binding to 29c[a].

entry	t(°)	$[Zn^{++}]\times10^4$[b]	$k_1(sec^{-1})$[c]	$k_2(M^{-1}sec^{-1})\times10^{-4}$[d]
i	27.8	3.31[e]	11.7±0.5	3.5±0.2
ii	27.8	3.31[f]	11.0±0.8	3.3±0.3
iii	27.8	3.31[g]	10.1±0.9	3.0±0.3
iv	27.8	6.62	8.8±0.5	-
v	28.2	3.31	11.3±0.5	3.4±0.2
vi	28.2	2.21	13.1±0.5	-
vii	28.2	1.10[h]	9.6±0.4	-
viii	22.8	3.31	11.0±0.5	3.3±0.2
ix	17.8[j]	3.31	5.0±0.5	-
x	33.0	3.31	19.8±1.6	5.0±0.5
xi	42.6	3.31	29.3±1.5	9.0±0.5
xii	46.3	3.31	34.8±1.7	10.5±0.5

continued......

Table X (continued)

---

[a] Measured under pseudo-first order conditions [M]/[29c] ≥ 10 by looking at the optical change at 290 nm, wavelength of maximum difference between the spectra of 29c and 29c:Zn^{++} complex. The concentration of 29c was 2×10^{-5} M unless otherwise stated.

[b] $Zn(NO_3)_2$ was used.

[c] Pseudo-first order rate constant.

[d] Second order rate constant $k_2 = k_1/[Zn^{++}]$.

[e] The "pH" of the zinc solution was 5.3.

[f] The "pH" of the zinc solution was adjusted to 6.2 by addition of NaOH.

[g] The "pH" of the zinc solution was adjusted to 6.9 by addition of NaOH.

[h] The concentration of 29c was 1×10^{-5} M.

[j] The kinetics at this temperature was no longer first order. The observed rate seemed to have two components a fast and a slower one. The value reported for k_1 corresponds to the fast one.

It can be seen from the first three entries that the rate of binding is constant within experimental error in the pH range 5.3-6.9, indicating that the rate-determining step is relatively independent of the state of protonation of the imidazole.

Fig. 11 shows the dependence of the pseudo-first order rate constant with the zinc concentration (entries iv-vii in Table X).

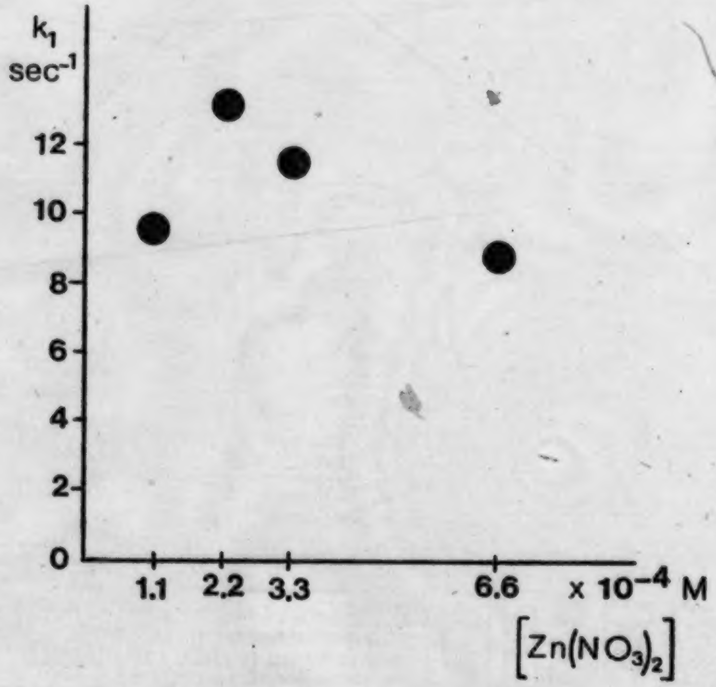


Fig. 11. Plot of the pseudo-first order rate constant of Zn^{++} binding to 29c vs. the concentration of $Zn(NO_3)_2$.

Contrary to expectations the rate of binding appears to be relatively independent of the zinc concentration. It is possible that the metal binds in a fast step to the first imidazole of the ligand, and then more slowly to the other two; the measured rate corresponding to this last rate determining step. If this were so, determining the rate of binding under conditions of successively lower [Zn^{++}], should ultimately alter the rate determining step to one dependent on [Zn^{++}].

The rate of binding was observed to increase with temperature. The Arrhenius plot (pH of the Zn^{++} solution:5.3) over a range of temperatures is shown in Fig. 12 (entries v, viii, x-xii in Table X).

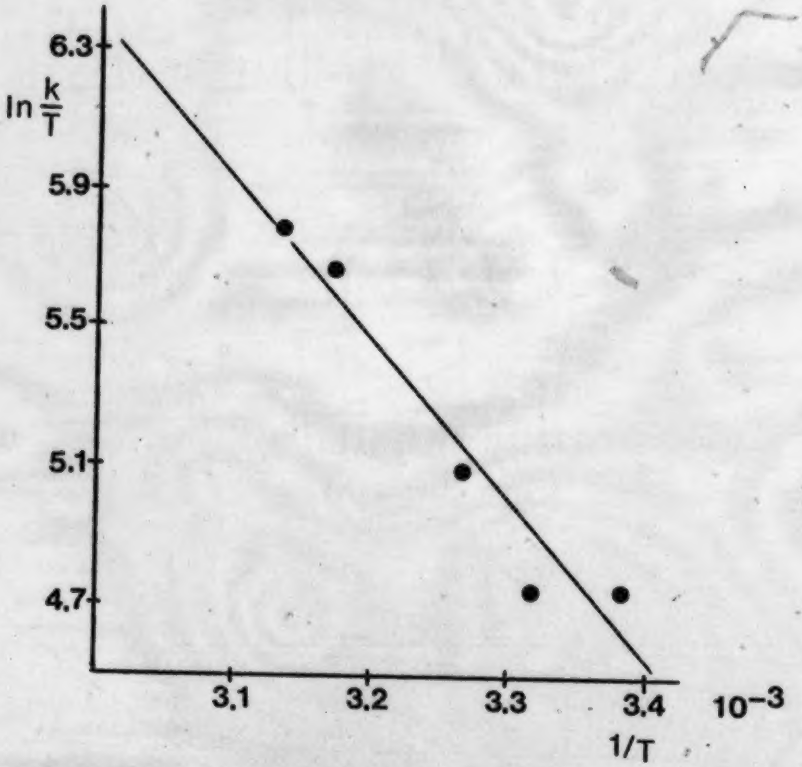


Fig. 12. Effect of temperature on the rate constant for the binding of Zn^{++} to 29c. The straight line is a least squares fit to the experimental data. $\ell n \frac{k}{T} = 20.49-4699.8 \left(\frac{1}{T}\right)$ r = 0.976

The energy and entropy of activation are 9.3 kcal mol^{-1} and -6.5 cal deg^{-1} mol^{-1} respectively. Although the rate of binding is quite slow (3.3×10^4 M^{-1} sec^{-1} at 23°) in agreement with 29c being a reasonable model for C.A. metal site, the activation parameters are more indicative of those for a smaller ligand. The very small activation entropy indicates that there is not very much solvent reordering in the rate determining step for the observed process.

5. Co(II) VISIBLE SPECTRA

For reasons explained in the previous chapter, Co(II) was used as a probe to assess the coordination geometry of the phosphine complexes.

U.V. spectra of 29a and 29b in the presence of $CoCl_2$ showed little if any evidence for four coordinate ligation[7b,65]. On the other hand, the di*iso*propylphosphine 29c in the presence of $CoCl_2$ showed reversible formation of a tetrahedral species (Fig. 13) at increasing pH with bands appearing at 588 (285), 622 (450), 646 (516), 662 (501) nm (ε)*, with an apparent "pK_a" (Fig. 14) around 5.5.

The 29c:Co(II) spectra were found to be highly

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*ε's were determined by adding known amounts of 29c to a cell containing 3 ml of 0.1 M $CoCl_2$ in 76% EtOH/H_2O.

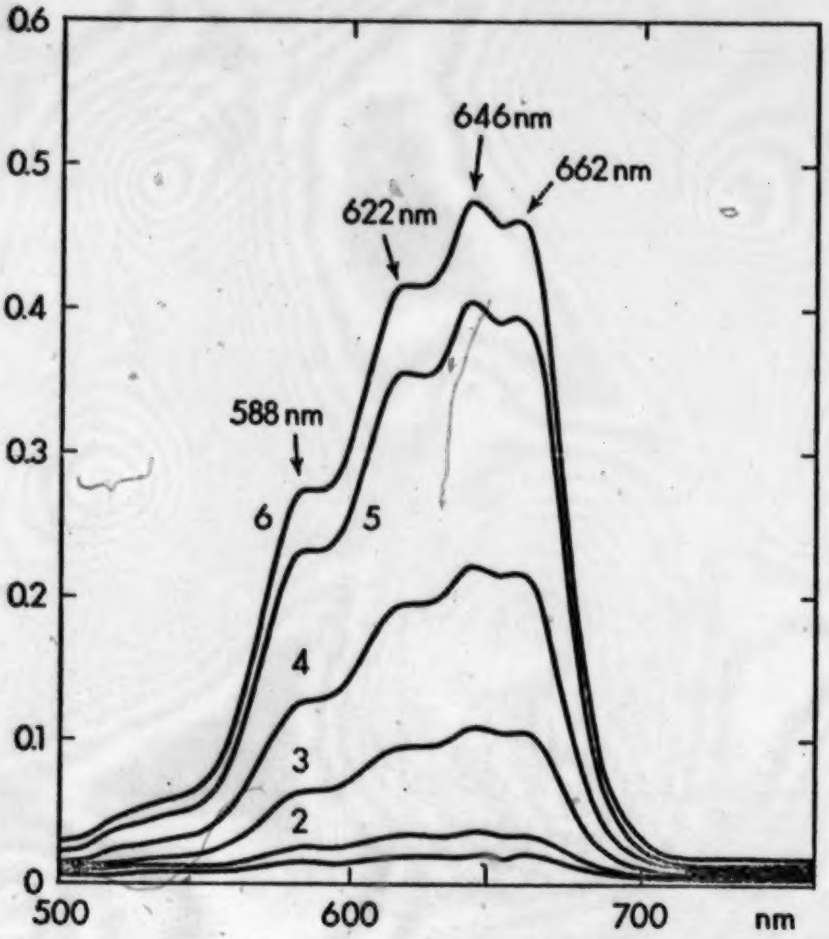


Fig. 13. UV-visible spectra of 29c:$CoCl_2$ complex at several pH's: 1, pH 3.8; 2, pH 4.1; 3, pH 4.7; 4, pH 5.2; 5, pH 6.05; 6, pH 7.45. (3 mL of 76% EtOH:H_2O, 0.01 mL of 1.0 M $CoCl_2$, 0.1 mL of 0.025 M 29c in EtOH, and pH adjusted with small additions of conc. aq. NaOH. Vertical scale, arbitrary units.

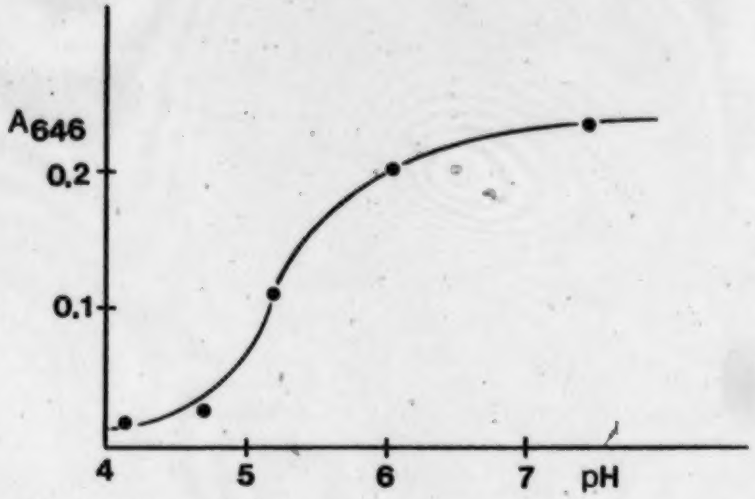


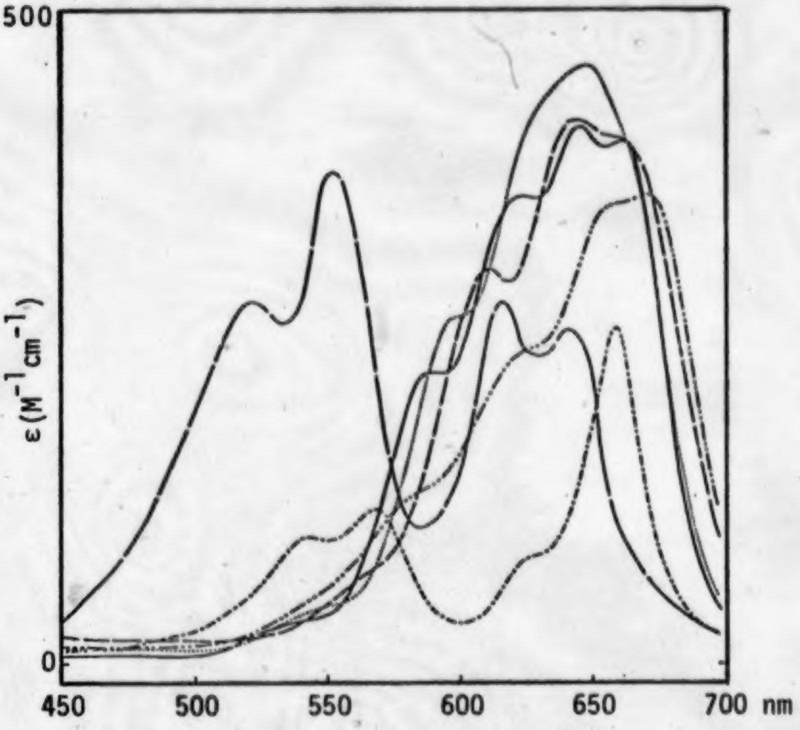
Fig. 14. Intensity of the 646 nm d-d transition of the complex 29c:$CoCl_2$ as a function of pH (from Fig. 13). The line drawn is an eye-guide only.

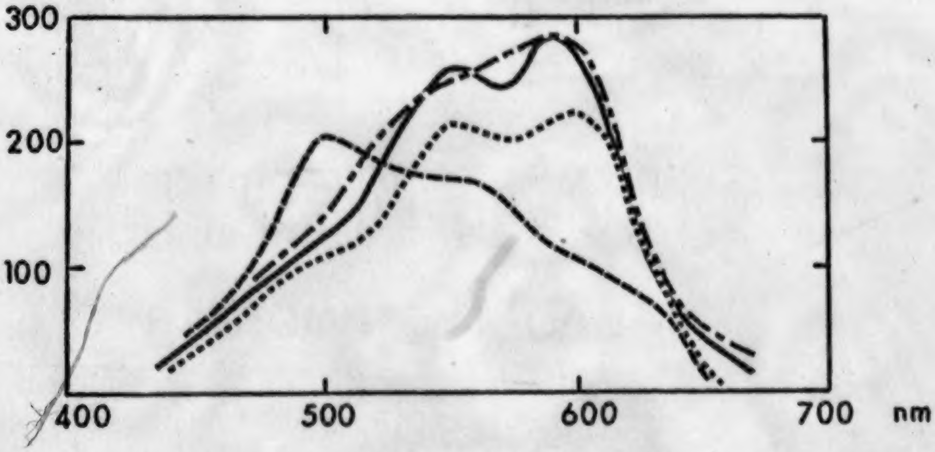
anion dependent (Fig. 15), reminiscent of the situation for the Co(II) enzyme (Fig. 16). In the presence of ClO_4^- and NO_3^- the 29c:Co(II) spectra were not indicative of a tetrahedral coordination but were more consistent with predominant 5 and/or 6 coordinate Co(II)[8a,10,65].

Thus the metal binding site of 29c is flexible enough to allow access of one or more additional ligands other than the three of the phosphine. This feature may be important if the transition state for CO_2 hydration by 29c:Zn^{++} involves five-coordinate zinc.

Fig. 15. U.V.-visible spectra of 1:1 29c:$Co(NO_3)_2$ solutions saturated with different anions in 76% ethanol:H_2O; Cl^- (—); Br^- (----); I^- (— — —); F^- (——-); N_3^- (—--—). The spectrum of Co(II) bovine carbonic anhydrase in unbuffered solution at pH 8.8 (— —) redrawn from ref. 11).

Fig. 16. Absorption spectra of the complexes of bovine Co(II) carbonic anhydrase with: Cl^- (—); Br^- (---); I^- (— —); N_3^- (—·—·—). Reproduced from refs. 8a and 7b.


500
0
ε(M⁻¹cm⁻¹)
450
500
550
600
650
700 nm


300
200
100
400
500
600
700
nm

6. CATALYTIC STUDIES

The catalytic activity of 29c towards PNPA hydrolysis and that of 29(a-c) towards CO_2 hydration were studied.

Compound 29c and its zinc complex proved to be inactive in catalyzing PNPA hydrolysis.

The results of the CO_2 hydration experiments are summarized in Table XI. Phosphines 29a and 29b at pH 7.5 show very small catalysis ($k_{cat} \approx 5\ M^{-1}\ sec^{-1}$) which does not seem to be enhanced by the addition of Zn^{++}. On the other hand, phosphine 29c shows a negligible catalytic effect at pH 7.5, but upon complexation with equimolar amount (10^{-3} M) Zn^{++} produces a catalytic rate enhancement of CO_2 hydration of $\approx 30\ M^{-1}\ sec^{-1}$. This catalytic rate enhancement is somewhat increased at pH 7.0 and it disappears completely at pH 6.5. This observation indicates that some basic form of the complex is probably the catalytically active species. In fact, titration experiments on 29c:Zn^{++} showed an ionization of some associated group which can be tentatively assigned as 29c:Zn^{++}-OH_2. However, the titration curve was not easily analyzed as having arisen from a single well-defined event. Probably the basic part of this titration was complicated by complex hydrolysis (similar to what happened to carbinol 17) due to the

TABLE XI

Rates of CO_2 hydration Catalyzed by Complexes 29:Zn^{++} at 25°.

	Reaction conditions[a]		$k_{obs}(sec^{-1})$	$k_{cat}(M^{-1}sec^{-1})$[b]
in 76% ethanol·water	Spontaneous	(pH 7.50)	0.175±0.015	
	29c	(pH 7.50)	0.170±0.020	c
	29c + Zn^{++}	(pH 7.50)	0.206±0.008	31±23
	Spontaneous	(pH 7.00)	0.386±0.008	
	29c + Zn^{++}	(pH 7.00)	0.491±0.035	105±43
	Spontaneous	(pH 6.50)	1.110±0.050	
	29c + Zn^{++}	(pH 6.50)	1.080±0.040	c
in water	Spontaneous	(pH 7.50)	0.0428±0.0014	
	29a	(pH 7.50)	0.0464±0.0004	3±2
	29a + Zn^{++}	(pH 7.50)	0.0469±0.0009	4±2
	29b	(pH 7.50)	0.0480±0.0020	5±4
	29b + Zn^{++}	(pH 7.50)	0.0500±0.0020	7±4

[a] Kinetics measured in 0.05 M HEPES buffer. Ionic strength was kept constant at 0.2 M with $NaClO_4$. pH values are those directly read from electrode immersed in solution. Due to solubility reasons, runs with 29a and 29b were performed in H_2O solutions, and those with 29c in 76% ethanol-water. When used, the catalyst concentration was 10^{-3} M. $Zn(ClO_4)_2$ was used as source of Zn^{++}.

[b] $k_{cat} = (k_{obs} - k_{spont})/[cat]$

[c] Negligible within experimental error.

relatively weak zinc binding ability of the ligand.

The CO_2 hydration was truly a catalytic process and not simply a reaction of 29c with CO_2 to form the corresponding carbamate because the amount of H^+ liberated was nearly the same as the initial $[CO_2]$, which in turn was held at 5-10 times that of catalyst. Thus the catalytically active form was turning over[84].

7. CONCLUSION

1. As observed in the carbinols, the presence of *iso*-propyl groups in the 4 and 5 positions of the imidazole inhibits 2:1 $(L:M^{++}:L)$ binding.

2. Substitution of the carbinol group by phosphorous seems to eliminate the dehydration problems present in the analogous carbinol ligands.

3. Compound 29c binds zinc in a tridentate symmetrical way, using its three imidazole rings.

4. The rate of 29c binding to zinc is similar to that of the apoenzyme, but the activation parameters are more indicative of a small ligand.

5. The metal binding ability of 29c is not as good as one would like it to be, perhaps due to reasons explained at the beginning of this chapter. This probably leads to complex hydrolysis at high pH's, and the concentration of active catalyst does not seem to increase

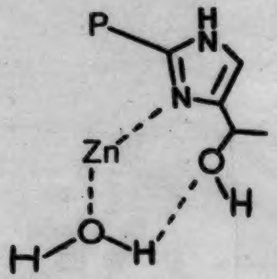
markedly by increasing the pH.

6. The UV-visible spectra of the 29cCo(II) complexes have several features in common with Co(II)-carbonic anhydrase.

7. Compound 29c constitutes the first model for the active site of C.A. which mimics at the same time several of the physicochemical properties of the enzyme, including some catalytic activity towards CO_2 hydration and bicarbonate dehydration.

8. Although this catalysis is encouraging, it is modest when compared to that exhibited by the enzyme. It is possible that simply creating a good metal-binding cavity is not sufficient to account for full enzymatic activity. Kannan et al[77] said that "it is also important to account for the role played by the system zinc-solvent-Thr 199 - Glu 106 in enzyme-catalzyed reactions when comparing and evaluating model systems for the carbonic anhydrases". Maybe the zinc-bound water needs to be hydrogen bonded in order to be fully efficient in hydrating CO_2.

9. At this point it can be suggested that the next step in the construction of a carbonic anhydrase model should be the incorporation of some secondary hydroxyl group(s) in the tridentate ligand, able to form hydrogen bond(s) with the water molecule coordinated directly to the metal, as in 30.



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This might lower the pK_a of this zinc-bound water, giving high concentrations of zinc-bound hydroxide at low enough pH's where the zinc ion is still tightly bound in the cavity of the ligand. Another advantage of the OH containing ligands might be their increased solubility in aqueous media, being then possible to study them at higher concentrations and as a consequence with higher accuracy.

III. EXPERIMENTAL

A. SYNTHESES

Routine IR, ^{1}H-nmr and exact mass spectra were recorded on a 7199 Nicolet FT IR spectrophotometer ($CHCl_3$ cast film), a Varian HA-100-15 spectrometer with Fourier Transform modifications provided by a Digilab FTS NMR-3 System, and an AEI MS-50 spectrometer, respectively.

4(5)-Vinylimidazole

It was prepared by the procedure of Overberger and Vorchheimer[49], mp 81-83° (lit.[49] mp 83.2-84.5°).

Copoly[N-vinylpyrrolidinone-4(5)-vinylimidazole]

The copolymer of N-vinylpyrrolidinone and 4(5)-vinylimidazole was prepared according to Kopple's procedure[47]. Polymer units with molecular weight $\geq$ 100000 were obtained by filtering a water solution of the crude polymer mixture through a XM 100 DIAFLO® membrane in a pressure filtration cell. The solution containing the desired size polymer was then lyophilized.

Anal. found: C, 62.52; H, 8.03; N, 12.40; O, 14.69 corresponding to (equation 14) 0.21 moles of imidazole per 100 g of polymer.

Tris(2-pyridyl)carbinol (9)

Compound 9 was prepared as described by Wibaut et al[50], mp 128-129° (lit.[50] 127-128°).

Bis(2-pyridyl)-2-(N-methylimidazolyl)carbinol (10)

A solution of 0.9 g (0.11 mol) of N-methylimidazole[51a] in 50 mL dry ether under N_2 was cooled to -30° (suspension) and treated with 7.5 mL of 1.6 m n-butyllithium in hexane, followed by stirring at room temperature for 30 min. It was then cooled to -40° and a solution of *bis*-2-pyridyl ketone[50] in 5 mL ether and 5 mL THF was added dropwise. After stirring at room temperature overnight the mixture was quenched with H_2O and the product was isolated by chloroform extraction. The combined extracts were dried ($MgSO_4$), and stripped of solvent to give a paste which was decolorized with charcoal and recrystallized from Skelly B/benzene to give 1.8 g (61%) of white crystals: mp 153-155°; IR 3350 (br), 1590, 1430, 1360, 1100 cm^{-1}; NMR (D_2O) Table VI; mass spectrum m/e 266 (M^+), 249 (M^+-OH); Anal. calcd for $C_{15}H_{14}N_9O$: C, 67.67; H, 5.26; N, 21.05. Found: C, 67.98; H, 5.37; N, 21.07.

Bis(2-pyridyl-6d)-2-(N-methylimidazolyl)carbinol (d-10)

It was prepared analogously to 10 but using *bis*-

(2-pyridyl-6d)ketone.

NMR (D_2O) Table VI; mass spectrum m/e 268 (M^+) 251 (M^+-OH).

Bis(2-pyridyl-6d)ketone

A solution of 21 g (0.132 mol) of 2-bromopyridine-6d in 300 mL dry ether under N_2 was treated with 90 mL of 1.6 M n-butyllithium in hexane at -65°. After two cycles of allowing the solution to warm to -45°, and recooling to -65°, 8 mL (0.067 mol) of diethylcarbonate was added keeping the temperature at -60°. Stirring was continued for 2 h at -65° and then at room temperature overnight. Workup consisted of the addition of 8 g H_2SO_4 in 10 mL H_2O, followed by ether extraction. The combined extracts were dried (Na_2SO_4), and stripped of solvent to give a dark oil which after distillation gave a yellow oil, bp 120-140°/0.15 Torr. Crystallization from toluene at -60° afforded 6 g (24%) of off-white crystals, mp 51-53°; IR 1680, 1575, 1430, 1320, 1300 cm^{-1}; ^1H-NMR ($CDCl_3$) δ 7.5 (2H, dd), 7.91 (2H, dd), 8.13 (2H, dd); mass spectrum m/e 186 (M^+), 79 (M^+-d-Pyr-C=O)*.

2-bromopyridine-6d

6-bromo-2-lithiopyridine[52] was quenched at -40°

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*Reference 50 reports the preparation of *bis*(2-pyridyl)-ketone in 10% yield from 2-pyridyl lithium and ethyl picolinate.

with an excess of methanol-d_1, and the mixture after workup, afforded 2-bromopyridine-6d, in 82% yield. ^1H NMR showed complete absence of the 2-H.

Tris(2-pyridyl-6d)carbinol (d-9)

In a procedure analogous to that described by Wibaut et al[50] for the preparation of 9, 2-lithiopyridine-6d and *bis*(2-pyridyl-6d)ketone were reacted to give 41% of d-9, mp 128-129° (lit.[50] 127-128° for 9); ^1H-NMR (D_2O) Table VI; mass spectrum m/e 266 (M^+), 249 (M^+-OH), 187 (M^+-6d pyr).

Bis(2-N-methylimidazolyl)-2-pyridyl carbinol (11)

To a suspension of 0.5 mol of 2-N-methylimidazolyl lithium in 600 mL dry ether at 0° was added 0.25 mol of ethyl picolinate and the mixture was stirred four hours. It was then decomposed with 20 mL of 10% H_2SO_4 and extracted with chloroform. The combined extracts were dried ($MgSO_4$) and solvent removed to give an oil from which unreacted N-methyl imidazole was removed by distillation (55°/3 Torr). The residue was recrystallized from Skelly B/benzene to give 6 g (9%) of 11, mp 131-133°; ^1H-NMR ($CDCl_3$) Table VI; mass spectrum m/e 269 (M^+), 191 (M^+-pyr); Anal. calcd. for $C_{14}H_{15}N_5O$: C, 62.45; H, 5.57; N, 26.02. Found: C, 62.80; H, 5.75; N, 26.12.

Bis(2-pyridyl)-2-(N-ethoxymethyl)imidazolyl carbinol (13b)

To a solution of 0.04 mol of 2-(N-ethoxymethyl)-imidazolyl lithium[44] in 200 mL THF at -60° was added a solution of 7.36 g (0.04 mol) of *bis*(2-pyridyl)ketone in 50 mL THF. After addition the deep blue solution was stirred an additional hour at -60° and then overnight at room temperature. Quenching with H_2O, followed by several ethyl acetate extractions which were combined and dried ($MgSO_4$) and then stripped of solvent yielding an oily paste which was recrystallized from ether to give 4.5 g (36%) of the product, mp 62-63.5°; ^1H-NMR ($CDCl_3$) δ 0.95 (3H, t), 3.20 (2H, q), 5.20 (2H, s), 7.07 (2H, d), 7.25 (3H, m), 7.75 (4H, m), 8.50 (2H, d).

Bis(2-pyridyl)-2-imidazolyl carbinol (13)

Deprotection of 13b (see above) was accomplished according to published procedure[44], and the free base liberated by basification, mp 225-235° (decomp.): ^1H-NMR (D_2O) Table VI; IR 3200 (br), 1570, 1430, 744 cm^{-1}; mass spectrum m/e 252 (M^+), 235 (M^+-OH), 185 (M^+-imidazolyl), 146 (M^+-pyr). Anal. calcd. for $C_{14}H_{12}ON_4 \cdot 1/2\ H_2O$: C, 64.4; H, 4.98; N, 21.4. Found: C, 64.70; H, 4.70; N, 21.16.

N-methoxymethyl-4,5-dimethylimidazole

This compound was prepared according to published procedure[51a] from 4,5-dimethylimidazole[78] in 24% yield, bp 68° (0.4 Torr); ^{1}H-NMR ($CDCl_3$) δ 2.22 (6H, s), 3.23 (3H, s), 5.15 (2H, s), 7.46 (1H, s); mass spectrum m/e 140 (M^+), 109 (M^+-OCH_3), 95 (M^+-CH_2OCH_3).

Tris [2-(N-ethoxymethyl)-4,5-dimethylimidazolyl]-carbinol (14b)

From 15.4 g (0.091 mol) of 2-(N-ethoxymethyl-4,5-dimethylimidazolyl) lithium reacted with 3.6 g (0.03 mol) diethyl carbonate in 200 mL THF at -60° was isolated 7.5 g crude orange crystals (50%) which were recrystallized from hexane/ether to give 14b, mp 114-116.5°: 90 MHz, ^{1}H-NMR ($CDCl_3$) δ 0.98 (3H, t), 2.03 (3H, s), 2.15 (3H, s), 3.15 (2H, q), 5.13 (2H, 5), 6.31 (1H, br); mass spectrum m/e 488 (M^+) 443 (M^+-OCH_2CH_3) 429 (M^+-$CH_2OCH_2CH_3$). The free ligand (14) was liberated analogously to published procedure[44] followed by basification, mp >275° (darkens): ^{1}H-NMR (D_2O) δ 2.26 (s). Anal. calcd. for $C_{25}H_{40}N_6O_4$: C, 61.45; H, 8.25; N, 17.20. Found: C, 61.27; H, 8.15; N, 17.44.

N-ethoxymethyl-4,5-di*iso*propylimidazole

This compound was prepared according to published procedure[44] from 4,5-di*iso*propylimidazole[78] in 34% yield, bp 95° (0.07 Torr); ^{1}H-NMR ($CDCl_3$) δ 1.30 (15H, m), 3.05 (2H, m), 3.43 (2H, q), 5.22 (2H, s), 7.40 (1H, s); mass spectrum m/e 210 (M^+), 155 ($M^+-OCH_2CH_3$), 141 ($M^+-CH_2OCH_2CH_3$).

Tris[2-(N-ethoxymethyl)-4,5-di*iso*propylimidazolyl]-carbinol (15b)

From 13.5 g (0.064 mol) of 2-lithio-N-ethoxymethyl-4,5-di*iso*propyl imidazole and 2.40 mL (0.021 mol) of diethyl carbonate was isolated 8.5 g (62%) of 15b (recryst. from ethyl acetate), mp 121-123°: ^{1}H-NMR ($CDCl_3$) δ 1.15 (45H, m), 3.05 (12H, m), 5.20 (6H, s), 6.75 (1H, br): mass spectrum m/e 656 (M^+), 639 (M^+-OH), 597 ($M^+-CH_2OCH_2CH_3$). The free base (15) was isolated analogously as for 14: ^{1}H-NMR ($CDCl_3$) δ 1.26 (36H, d), 3.01 (6H, hept.). Anal. calcd. for $C_{28}H_{46}N_6O$: C, 69.70; H, 9.54; N, 17.43. Found: C, 69.33; H, 9.54; N, 17.49.

N-methyl-4,5-di*iso*propylimidazole

A suspension of 10.9 g (0.0722 mol) of 4,5-di*iso*propylimidazole[78] in 500 mL THF was treated at -10° with 32.8 mL of 2.2 M *n*-butyllithium in hexane, and stirred for 15 min until almost all solids dissolved. Then 4.49 mL of methyl iodide were added. After workup and ether extraction an oil was obtained which was purified by distillation to give 8.8 g (68%) of product: bp 43-45° (0.05 Torr); ^{1}H-nmr ($CDCl_3$) δ 1.37 (12H, dd), 3.0 (2H, m), 3.55 (3H, s), 7.25 (1H, s); mass spectrum m/e 166 (M^+), 151 (M^+-CH_3).

Tris-2-(N-methyl-4,5-di*iso*propylimidazolyl)-carbinol (16)

From 0.058 mol of 2-(N-methyl-4,5-di*iso*propylimidazolyl)lithium and 0.019 mol diethylcarbonate was isolated ~50% of an oil containing 16 and its analogous ketone. One gram of this mixture was chromatographed over silica gel (ethylacetate) to give the desired 16 as an oil: ^{1}H-NMR (acetone-d_6) δ 1.15 (18H, d), 1.32 (18H, d), 3.5 (6H, m), 3.27 (9H, m), 6.42 (1H, s, br). Anal. calcd. for $C_{31}H_{52}N_6O$: C, 70.99; H, 9.92; N, 16.03. Found: C, 71.17; H, 10.04; N, 15.64.

N-methoxymethyl-2,4,5-trimethylimidazole

A solution of the lithium salt of N-methoxymethyl-4,5-dimethylimidazole (0.071 mol) in 250 mL THF was treated with 0.071 mol of methyl iodide. After work-up and ether extraction a yellow oil was obtained which was purified by distillation to give the desired product in 90% yield: bp 50-53° (0.03 Torr); ^{1}H-NMR ($CDCl_3$) δ 2.12 (6H, s), 2.38 (3H, s), 3.25 (3H, s), 5.08 (2H, s); mass spectrum m/e 154 (M^+), 123 (M^+-OCH_3), 109 ($M^+-CH_2OCH_3$).

Bis-2-[N-methyl-4,5-di*iso*propylimidazolyl]ketone (16b)

Heating of neat 16 at 180°/Torr for 5 min yielded an oil which was recrystallized from ether, mp 197-199°; ^{1}H-NMR ($CDCl_3$) δ 1.23 (12H, d), 1.34 (12H, d), 3.1 (4H, m), 3.85 (6H, s); mass spectrum, m/e 358 (M^+), 343 (M^+-CH_3); Anal. calcd. for $C_{21}H_{34}N_4O$: C, 70.39; H, 9.49; N, 15.64. Found: C, 70.23; H, 9.50; N, 15.60.

Bis-2-[N-methyl-4,5-di*iso*propylimidazolyl]-2-[N-methoxymethyl-4,5-dimethylimidazolylmethyl]-carbinol, 17

To 1.29 g (0.0084 mol) N-methoxymethyl-2,4,5-trimethylimidazole in 180 mL dry THF at -60° was added 1 eq. (0.0084 mol) n-BuLi in hexane. To this mixture was added 3.09 g (0.0084 mol) of *bis*-2-[N-methyl-4,5-di*iso*propylimidazolyl]ketone in 10 mL THF. After workup, and recrystallization from ether, 2.7 g (63%) of the product was obtained, mp 133-134°. ^{1}H-NMR ($CDCl_3$) δ 1.11 (12H, d), 1.26 (12H, d), 1.98 (3H, s), 2.09 (3H, s), 2.93 (4H, m), 3.23 (9H, d), 3.87 (2H, s), 5.35 (2H, s); mass spectrum, m/e 512 (M^+), 497 (M^+-CH_3) 359 (M^+-N-methoxymethyl-4,5-dimethylimidazolylmethyl). Anal. calcd. for $C_{29}H_{48}N_6O_2$: C, 67.83; H, 9.35; N, 16.37. Found: C, 68.03; H, 9.42; N, 16.51.

The free base (17) was obtained by refluxing the above material in 10% HCl for two weeks and basification, mp 168-170° (darkens). ^{1}H-NMR ($CDCl_3$) δ 1.23 (24H, d), 2.10 (6H, s), 2.93 (10H, m), 3.57 (2H, s); mass spectrum, m/e 468 (M^+), 450 (M^+-H_2), 359 (M^+-4,5-dimethylimidazolylmethyl).

Tris-2-[4,5-di*iso*propylimidazolyl]methane (20)

Under nitrogen, 100 mg of the *tris*-2-[4,5-di*iso*propylimidazolyl]carbinol xHCl salt was dissolved in 10 mL 3N NaOH and 10 mL ethanol and brought to reflux, before 100 mg of sodium dithionite dissolved in the minimum amount of H_2O was added in one portion. After one minute, the mixture was acidified with conc. HCl, and then all volatiles were removed under vacuum. The residue was triturated with acetone which was filtered and evaporated to afford a yellowish solid, mp 120-180° (decomp.), mass spectrum, m/e 466 (M^+-xHCl), 451 (M^+-xHCl-CH_3).

This material proved to be relatively stable as the HCl salt but upon basification in the presence of air discolored badly due to air oxidation followed by dehydration.

The phosphines 29(a-c) were kindly prepared by Dr. R.S. Brown using the method described in ref. 72.

Tris[2-(N-methylimidazolyl)]carbinol (12)

This compound was prepared as reported by Tang et al[44], mp 178-179° (lit.[44] 177.5-179.5°).

B. POTENTIOMETRIC TITRATIONS

1. pK_a DETERMINATIONS

These were performed in a jacketed cell kept at 30 ± 0.1°. Air was excluded from the cell by passing a gentle stream of nitrogen (purified by passing successively through a solution of $Ba(OH)_2$ and of water) through the cell. The pH was measured using a Radiometer TTT2 titrator and PHA 943 B titration module in conjunction with a Radiometer 6K2402B combined electrode, and recorded as a function of added 0.1000 NaOH (delivered by a Radiometer ABU 12 autoburette), on a Radiometer SBR 3 titrigraph. Standard pH 4 and pH 7 buffers were used to check the electrode linearity and standardize the pH meter at the beginning of each series of experiments. The ionic strength was maintained constant by using a medium 0.16 M in KNO_3. Data were analyzed by a computer version of the Simm's method[55] (APPENDIX I) and reported pK_a's are the average of at least three determinations.

2. METAL BINDING CONSTANTS

Stock solutions of Co^{++}, Ni^{++}, Zn^{++}, and Cu^{++} were prepared from their reagent grade hydrated nitrate salts and were standardized by EDTA titration[82]. Typically a three to four-fold excess of ligand over metal was titrated as above and the data analyzed (APPENDIX II) to give metal binding constants which are reported as the average of three determinations.

3. COMPLEX TITRATIONS

A solution consisting of an equimolar amount of ligand and metal, and a known amount of strong acid was titrated with NaOH, following the same method as above. After all the acid added had been titrated, and when the consumption of one extra equivalent (based on the amount of complex present) was clear, the pK_a of the ionizable group was determined graphically as the pH at which an extra half equivalent of OH^- had been consumed. Typically 1 mL of 0.025 M ligand, the stoichiometric amount of metal added as ca. 0.01 M solution, 3 mL of 0.25 M KNO_3, and 1 mL of 0.1050 M HNO_3 were mixed in the thermostated titration cell and titrated with 0.1000 M NaOH.

C. NUCLEAR MAGNETIC RESONANCE STUDIES OF Zn^{++} COMPLEXES

Typically 2-8 mg of ligand were dissolved in ca. 0.35 mL of D_2O and microliter amounts of 0.5 M $ZnBr_2$ in D_2O were added, and spectra recorded after each addition. When needed, methanol-d_4, acetone-d_6 or dimethylsulfoxide-d_6 were added to solubilize the complex.

D. RATES OF Zn^{++} BINDING TO 29c

These were studied under pseudo-first order conditions ($[Zn^{++}]/[\underline{29c}] \geq 15$) in 76% ethanol-water. In a typical experiment, a 3.31×10^{-4} M $Zn(NO_3)_2$ solution and a 2×10^{-5} M 29c solution were mixed in an Aminco-Morrow stopped-flow system, and the binding monitored as transmittance change at 290 nm. The data were analyzed by an analog comparison technique. The transmittance-time curves were stored on a Tracor NS-570 signal averager and then output to a dual-trace oscilloscope for comparison to a synthetic exponential decay curve whose time constant could be changed by changing the resistance in the circuit. The temperature was kept constant by means of a standard temperature control system. The enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation were obtained by plotting $\ell n(k_2/T)$ versus $1/T$ (Fig. 12). According to equation 30 (ref. 79)

$$k_2 = \frac{kT}{h} \exp\left(-\frac{\Delta H^{\ddagger}}{RT}\right) \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \tag{30}$$

where k_2 is the pseudo-first order rate constant, k the Boltzmann constant, h Planck's constant, and R and T have their usual meanings, the slope of such plot is $-\Delta H^{\ddagger}/R$ and the intercept $\ell n(k/h) + \Delta S^{\ddagger}/R$.

E. CATALYTIC STUDIES

1. HYDROLYSIS OF PNPA

p-Nitrophenyl acetate was prepared as described by

Chattaway[80]. After two recrystallizations from Skelly B the product was nearly colorless, mp 76-78° (lit.[81] mp 79.5-80°).

A stock solution of PNPA (1.00×10^{-2} M) in 98% ethanol was kept tightly stoppered and refrigerated. The kinetic runs were performed on a Unicam SP1800 recording spectrophotometer equipped with a Unicam AR25 linear recorder. The cell compartments were thermostated at 30 ± 0.1°, and hydrolysis experiments were carried out using 1 cm path-length, 3 mL capacity quartz cuvettes. In a typical run, 3 mL of buffer was introduced to the cuvettes, followed by 1 μL of 10 M $ZnCl_2$ solution and 40 μL of 0.245 M ligand solution. The resulting concentration of complex was approximately 3×10^{-3} M. The reaction was initiated by adding a small aliquot (20 μL) of PNPA stock solution to the cuvette and mixing. The reactions were followed by the increase in absorbance at 400 nm (λ_{max} of *p*-nitrophenolate anion) for at least three half-lives, and the infinity absorbance was taken after 7-10 half-lives. At the end of the run, the pH of the reaction mixture was measured. The reading generally changed by no more than 0.05 unit during the course of a run. The pseudo-first order hydrolysis rate constant was calculated by a non-linear least squares program*. Control experiments were run simultaneously in cells containing ligand but no metal, and metal but no ligand respectively.

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*This program was kindly provided by Prof. R.E.D. McClung.

2. HYDRATION OF ACETALDEHYDE

The first order disappearance of acetaldehyde was followed by monitoring the decrease in the carbonyl absorption at 276 nm (ε 16 M^{-1} cm^{-1}), according to the method of Prince and Woolley[83]. All experiments were conducted in the same apparatus as for PNPA hydrolysis but at 0.0 ± 0.2°, the cell mounting being cooled by a water-ethyleneglycol mixture from a thermostated bath. The absorbance was set to 0.5 units full scale, and the chart speed of the recorder to 2-10 sec/cm. With a 1 cm quartz cell filled with 3.0 mL of thermally equilibrated buffer solution containing the catalyst to be studied in the light beam, the pen recorder's adjustable scale was set to read 0.0 on the chart. A known volume, usually 5 µL, of acetaldehyde was then introduced rapidly from a chilled microsyringe; the solution was mixed and the chart paper set in motion. Readings could be taken from the chart within less than 10 sec of acetaldehyde addition.

3. CO_2 HYDRATION AND BICARBONATE DEHYDRATION[66]

A solvent consisting of 76% ethanol-water was used for kinetic runs with 29c and 17, and water for 29a and 29b. A CO_2 stock solution was prepared by bubbling the pure gas into the solvent for at least 30 min at room temperature. Solutions of lower concentrations were prepared by dilution of the stock solution. To determine the concentration of CO_2, a known volume of the saturated solution was added to an excess of standardized $Ba(OH)_2$ containing $BaCl_2$. The resulting solution was back-titrated against standardized HCl with phenolphthalein as the indicator. Solutions of $NaHCO_3$ were prepared by carefully weighing the salt and dissolving it in freshly boiled solvent just prior to use. The buffer used was a 0.05 M HEPES solution containing 10^{-3} M $Zn(ClO_4)_2$, 10^{-3} M ligand, 10^{-4} M *p*-nitrophenol, and enough sodium perchlorate (ca. 0.15 M) to make the ionic strength equal to 0.2 M, and the resulting solution adjusted to the required pH with concentrated sodium hydroxide. A stock $Zn(ClO_4)_2$ solution (0.0948 M) was prepared from solid zinc carbonate and perchloric acid, and its zinc content determined by EDTA titration[82]. Kinetic experiments were performed on an Aminco-Morrow stopped-flow spectrophotometer. The CO_2 solution obtained by dilution of the stock solution with solvent containing 0.2 M $NaClO_4$ was placed in one syringe, and a solution containing the complex, indicator and buffer in the other syringe. The CO_2 hydration reaction was followed at 400 nm (*p*-nitrophenolate anion). The total change in transmittance did not exceed 5%, and the pH of the solution after mixing usually fell to a value near to 0.1 pH unit lower than the initial pH. The oscilloscope trace readings were used to determine first order hydration rate constants[46] by comparison to a synthetic exponential curve, using the same electronic set-up as described for the rate of binding of Zn^{++} to 29c. Control experiments in the absence of metal, and in the absence of ligand and metal (spontaneous) were also performed.

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APPENDIX I

pK_a DETERMINATION BY POTENTIOMETRIC TITRATION

Let L be an organic base that can accept three protons according to the following equations:

$$L + H \rightleftharpoons LH \qquad (AI.1)$$

$$LH + H \rightleftharpoons LH_2 \qquad (AI.2)$$

$$LH_2 + H \rightleftharpoons LH_3 \qquad (AI.3)$$

H, LH, LH_2, and LH_3 represent H^+, LH^+, LH_2^{++}, and LH_3^{+++} respectively. Charges are omitted for simplicity.

Let us define the dissociation constants:

$$K_{a1} = [L][H]/[LH] \quad ; \quad pK_{a1} = -\log K_{a1} \qquad (AI.4)$$

$$K_{a2} = [LH][H]/[LH_2] \quad ; \quad pK_{a2} = -\log K_{a2} \qquad (AI.5)$$

$$K_{a3} = [LH_2][H]/[LH_3] \quad ; \quad pK_{a3} = -\log K_{a3} \qquad (AI.6)$$

Let us define $\bar{n}_H$ as the average number of hydrogen ions bound per molecule of base:

$$\bar{n}_H = ([LH] + 2[LH_2] + 3[LH_3])/([L] + [LH] + [LH_2] + [LH_3]) \qquad (AI.7)$$

If the pK_a's are different enough:

When $pH = pK_{a1}$

$$[L] = [LH] \text{ and } [LH_2], [LH_3] << [LH]$$

Then, from AI.7

$$\bar{n}_H = [LH]/([L] + [LH]) = 0.5 \qquad (AI.8)$$

When pH $\approx$ pK_{a2}

$$[LH] \approx [LH_2] \text{ and } [L], [LH_3] \ll [LH]$$

Then, from AI.7

$$\bar{n}_H = ([LH] + 2[LH_2])/([LH] + [LH_2]) = 1.5 \qquad (AI.9)$$

When pH = pK_{a3}

$$[LH_2] \approx [LH_3] \text{ and } [L], [LH] \ll [LH_2]$$

Then, from AI.7

$$\bar{n}_H = (2[LH_2] + 3[LH_3])/([LH_2] + [LH_3]) = 2.5 \qquad (AI.10)$$

From AI.8, AI.9, and AI.10, pK_{a1}, pK_{a2}, and pK_{a3} will be the pH values of the solution when $\bar{n}_H$ is 0.5, 1.5, and 2.5 respectively.

It is only needed to express $\bar{n}_H$ as a function of experimentally determinable variables:

$$\bar{n}_H = \frac{[H]_T - [H] - [OH^-]_{added} + \frac{K_W}{[H]}}{[L]_T} = \frac{C_S}{[L]_T} \qquad (AI.11)$$

where $[H]_T$ = total concentration of strong acid added at the beginning.

$[H]$ = measured as pH.

$[L]_T$ = total concentration of organic base present.

K_W = dissociation constant of water 1.66×10^{-14}.

The program "ionisation" computes $\bar{n}_H$ for each addition of NaOH.

```
#1 ionisation
>     C      IONISATION CONSTANTS
>            REAL NH
>     1      FORMAT(I2)
>     2      FORMAT(2F10.6)
>     5      FORMAT('  IONISATION CONSTANTS')
>      6      FORMAT(/'VOLUME',4X,'PH',7X,'NH')
>     7      FORMAT(/F6.4,4X,F5.2,5X,E10.4)
>     9      FORMAT(4F10.7)
>     13     FORMAT(//'   CNAOH',12X,'CLH',13X,'CL',11X,'VOLI')
>     14     FORMAT(1F10.6,5X,F10.6,5X,F10.6,5X,F10.6)
>            READ(5,1)M
>            DO 110 I=1,M
>            READ(5,9)CNAOH,CLH,CL,VOLI
>            WRITE(6,5)
>            WRITE(6,6)
>            READ(5,1)N
>            DO 100 J=1,N
>            READ(5,2) VOLUME,PH
>            FF=10**(-PH)
>            VOL=VOLUME+VOLI
>            CORRT=1.66E-14/FF
>            CS=CLH-(CNAOH*VOLUME+FF*VOL)+CORRT*VOL
>            NH=CS/CL
>     100    WRITE(6,7)VOLUME,PH,NH
>            WRITE(6,13)
>            WRITE(6,14)CNAOH,CLH,CL,VOLI
>            STOP
>     110    CONTINUE
>            END
#END OF FILE
```

DATA:

M - number of runs

CNAOH - concentration of sodium hydroxide used in the titration (M)

CLH - mmoles of strong acid added at the beginning

CL - mmoles of organic base present

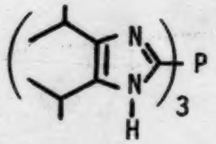
VOLI - initial volume

N - number of pairs (VOLUME, PH)

VOLUME - volume of NaOH added

PH - pH of solution after the addition

Example: Program output for pK_a's determination.

of 

```
   VOL          PH          nH
  .000         2.40        3.40
  .100         2.48        3.12
  .200         2.60        2.88
  .300         2.73        2.60
  .320         2.76        2.55
  .340         2.79        2.49
  .360         2.83        2.44
  .380         2.87        2.39
  .400         2.92        2.34
  .500         3.20        2.06
  .600         3.80        1.76
  .620         3.95        1.69
  .640         4.09        1.62
  .660         4.23        1.55
  .680         4.35        1.47
  .700         4.50        1.39
  .800         5.28        1.00
  .900         6.38         .60
  .920         6.52         .52
  .940         6.66         .44
  .960         6.78         .36
  .980         6.93         .28
 1.000         7.08         .20
```

Where:

CNAOH = 0.1000

CLH = 0.1050

CL = 0.0250

VOLI = 5.00

The values of pH when $\bar{n}_H$ = 0.5, 1.5, and 2.5 can be obtained by any interpolation method.

APPENDIX II

STABILITY CONSTANTS OF METAL COMPLEXES AS DETERMINED BY POTENTIOMETRIC TITRATION

Let L be an organic ligand that can form complexes with metal ions according to the following equations:

$$M + L \rightleftharpoons ML \qquad \text{(AII.1)}$$

$$ML + L \rightleftharpoons ML_2 \qquad \text{(AII.2)}$$

M, ML, and ML_2 represent M^{++}, ML^{++}, and ML_2^{++}. Charges are omitted for simplicity. The stepwise dissociation constants can be defined as:

$$K_1 = [M][L]/[ML] \quad ; \; pK_1 = -\log K_1 \qquad \text{(AII.3)}$$

$$K_2 = [ML][L]/[ML_2] \; ; \; pK_2 = -\log K_2 \qquad \text{(AII.4)}$$

Let $\bar{n}$ be the average number of base molecules bound per metal ion:

$$\bar{n} = ([ML] + 2[ML_2])/([M] + [ML] + [ML_2]) \qquad \text{(AII.5)}$$

If pK_1 and pK_2 are different enough:

When $pL = pK_1$

$[M] = [ML]$ and $[ML_2] << [ML]$

Then, from AII.5

$$\bar{n} = [ML]/([M] + [ML]) = 0.5 \qquad \text{(AII.6)}$$

When $pL = pK_2$

$[ML] = [ML_2]$ and $[M] << [ML]$

Then, from AII.5

$$\bar{n} = ([ML] + 2[ML_2])/([ML] + [ML_2]) = 1.5 \qquad \text{(AII.7)}$$

From AII.6 and AII.7, pK_1 and pK_2 are the pL values when $\bar{n}$ is 0.5 and 1.5 respectively. It is needed to express $\bar{n}$ and [L] as functions of experimentally determinable variables:

$$[L]_T = [L]_{free} + [L]_{prtonated} + [L]_{bound\ to\ metal}$$

or

$$[L]_T = [L] + [LH] + [LH_2] + [LH_3] + \bar{n}[M]_T \qquad \text{(AII.8)}$$

then

$$\bar{n} = \frac{[L]_T - ([L] + [LH] + [LH_2] + [LH_3])}{[M]_T} \qquad \text{(AII.9)}$$

Substituting AI.11 in AII.9

$$\bar{n} = \frac{[L]_T - \dfrac{C_S}{\bar{n}_H}}{[M]_T} \qquad \text{(AII.10)}$$

$[L]_T$ and $[M]_T$ are known, C_S can be obtained from experimental data (AI.11), and $\bar{n}_H$ can be expressed as a function of pH and the acid dissociation constants of the organic base:

$$\bar{n}_H = \frac{\dfrac{[L][H]}{K_{a1}} + 2\,\dfrac{[L][H]^2}{K_{a1}K_{a2}} + 3\,\dfrac{[L][H]^3}{K_{a1}K_{a2}K_{a3}}}{[L] + \dfrac{[L][H]}{K_{a1}} + \dfrac{[L][H]^2}{K_{a1}K_{a2}} + \dfrac{[L][H]^3}{K_{a1}K_{a2}K_{a3}}} =$$

$$= \frac{K_{a2}K_{a3}[H] + 2\,K_{a3}[H]^2 + 3\,[H]^3}{K_{a1}K_{a2}K_{a3} + K_{a2}K_{a3}[H] + K_{a3}[H]^2 + [H]^3} \qquad \text{(AII.11)}$$

Let us define $$\alpha = \frac{L}{[L] + [LH] + [LH_2] + [LH_3]} = \qquad \text{(AII.12)}$$

$$= \frac{K_{a1}K_{a2}K_{a3}}{K_{a1}K_{a2}K_{a3} + K_{a2}K_{a3}[H] + K_{a3}[H]^2 + [H]^3} \qquad \text{(AII.13)}$$

From AI.7 and AI.11 substituted in AII.12

$$[L] = \alpha([L] + [LH] + [LH_2] + [LH_3]) = \alpha \cdot \frac{C_S}{\bar{n}_H} \qquad \text{(AII.14)}$$

The program "stability" computes C_S, $\bar{n}_H$, α, [L], $\bar{n}$, and pL for each addition of NaOH.

```
#1.stabilitw
>   C      STABILITY CONSTANT
>   C
>            IMPLICIT REAL*8 (A-H,O-Z)
>            REAL*8 NL,NLL
>   1        FORMAT(I2)
>   2        FORMAT(F6.4,F4.2)
>   5        FORMAT('  STABILITY CONSTANT')
>   6        FORMAT(/' VOLUME',4X,'PH',7X,'CS',9X,'NLL',8X,'ALFA',11X,'RB',
>           *12X,'NL',10X,'PB')
>   7        FORMAT(/F6.4,4X,F5.2,5X,F6.4,4X,F6.3,4X,E10.4,4X,E10.4,4X,E10.4,
>           *4X,F7.4)
>   9        FORMAT(F10.4,F10.7,F10.7,F10.7,F10.4)
>   10       FORMAT(F10.4,F10.4,F10.4)
>   13       FORMAT(//'   CNAOH',12X,'CM',12X,'CLH',13X,'CL',11X,'VOLI',11X
>           *'PK1',12X,'PK2',12X,'PK3')
>   14       FORMAT(1F10.6,5X,F10.6,5X,F10.6,5X,F10.6,5X,F10.6,5X,F10.6,5X,
>           *F10.6,5X,F10.6)
>            READ(5,1)M
>            DO 110 I=1,M
>            READ(5,9) CNAOH,CM,CLH,CL,VOLI
>            READ(5,10) PK1,PK2,PK3
>            AK1=10.**(-PK1)
>            AK2=10.**(-PK2)
>            AK3=10.**(-PK3)
>            WRITE(6,5)
>            WRITE(6,6)
>            READ(5,1) N
>            DO 100 J=1,N
>            READ(5,2) VOLUME,PH
>            FF=10**(-PH)
>            VOL=VOLUME+VOLI
>            CORRT=1.66E-14/FF
>            CS=CLH-(CNAOH*VOLUME+FF*VOL)+CORRT*VOL
>            TT=AK3*AK2*FF+2*AK3*FF**2+3*FF**3
>            SB=AK1*AK2*AK3+AK2*AK3*FF+AK3*FF**2+FF**3
>            NLL=TT/SB
>            NL=(CL-CS/NLL)/CM
>            ALFA=AK1*AK2*AK3/SB
>            TANG=CS/VOL
>            RB=ALFA*TANG/NLL
>            PB=-DLOG10(RB)
>   100      WRITE(6,7) VOLUME,PH,CS,NLL,ALFA,RB,NL,PB
>            WRITE(6,13)
>            WRITE(6,14) CNAOH,CM,CLH,CL,VOLI,PK1,PK2,PK3
>            STOP
>   110      CONTINUE
>            END
#END OF FILE
```

DATA:

M - number of runs

CNAOH - concentration of NaOH used in the titration (M)

CM - mmoles of metal ion added at the beginning

CLH - mmoles of strong acid added at the beginning

CL - mmoles of organic base present

VOLI - initial volume

PK1 - pK_{a1} as defined in AI.4

PK2 - pK_{a2} as defined in AI.5

PK3 - pK_{a3} as defined in AI.6

N - number of pairs (VOLUME, PH)

VOLUME - volume of NaOH added

PH - pH of the solution after the addition

If the organic ligand has only one pK_a because it can only protonate once, equations AII.11 and AII.12 turn into:

$$\bar{n}_H = \frac{[H]}{K_{a1} + [H]} \qquad \text{(AII.15)}$$

$$\alpha = \frac{K_{a1}}{K_{a1} + [H]} \qquad \text{(AII.16)}$$

By making $pK_{a2} = pK_{a3} = 0$ equations AII.11 and AII.12 are good approximations for AII.15 and AII.16, because from AII.11 $\bar{n}_H = \frac{[H] + 2[H]^2 + 3[H]^3}{K_{a1} + [H]} \simeq \frac{[H]}{K_{a1} + [H]}$

from AII.12 $\alpha = \frac{K_{a1}}{K_{a1} + [H] + [H]^2 + [H]^3} \simeq \frac{K_{a1}}{K_{a1} + [H]}$

and the same program can still be used. If the organic ligand has two pK_a's, the expressions for $\bar{n}_H$ and α are:

$$\bar{n}_H = \frac{K_{a2}[H] + 2[H]^2}{K_{a1}K_{a2} + K_{a2}[H] + [H]^2} \qquad (AII.17)$$

$$\alpha = \frac{K_{a1}K_{a2}}{K_{a1}K_{a2} + K_{a2}[H] + [H]^2} \qquad (AII.18)$$

Substituting $pK_{a3} = 0$ in AII.11 and AII.12 one gets:

$$\bar{n}_H = \frac{K_{a2}[H] + 2[H]^2 + 3[H]^3}{K_{a1}K_{a2} + K_{a2}[H] + [H]^2 + [H]^3} \qquad (AII.19)$$

$$\alpha = \frac{K_{a1}K_{a2}}{K_{a1}K_{a2} + K_{a2}[H] + [H]^2 + [H]^3} \qquad (AII.20)$$

For some values of K_{a2} and [H], the term $[H]^3$ might not be negligible in AII.19 and AII.20. For this reason a new program "stability 2" is used to compute the right expressions AII.17 and AII.18.

```
#1 stability2
>    C     STABILITY CONSTANT
>    C
>          IMPLICIT REAL*8 (A-H,O-Z)
>          REAL*8 NL,NLL
>    1     FORMAT(I2)
>    2     FORMAT(F6.4,F4.2)
>    5     FORMAT('  STABILITY CONSTANT')
>    6     FORMAT(/' VOLUME',4X,'PH',7X,'CS',9X,'NLL',8X,'ALFA',11X,'RB',
>         *12X,'NL',10X,'PB')
>    7     FORMAT(/F6.4,4X,F5.2,5X,F6.4,4X,F6.3,4X,E10.4,4X,E10.4,4X,E10.4,
>         *4X,F7.4)
>    9     FORMAT(F10.4,F10.7,F10.7,F10.7,F10.4)
>    10    FORMAT(2F10.4)
>    13    FORMAT(//'   CNAOH',12X,'CM',12X,'CLH',13X,'CL',11X,'VOLI',11X
>         *'PK1',12X,'PK2',12X,'PK3')
>    14    FORMAT(1F10.6,5X,F10.6,5X,F10.6,5X,F10.6,5X,F10.6,5X,F10.6,5X,
>         *F10.6)
>          READ(5,1)M
>          DO 110 I=1,M
>          READ(5,9) CNAOH,CM,CLH,CL,VOLI
>          READ(5,10) PK1,PK2
>          AK1=10.**(-PK1)
>          AK2=10.**(-PK2)
>          WRITE(6,5)
>          WRITE(6,6)
>          READ(5,1) N
>          DO 100 J=1,N
>          READ(5,2) VOLUME,PH
>          FF=10**(-PH)
>          VOL=VOLUME+VOLI
>          CORRT=1.66E-14/FF
>          CS=CLH-(CNAOH*VOLUME+FF*VOL)+CORRT*VOL
>          TT=AK2*FF+2*FF**2
>          SB=AK1*AK2+AK2*FF+FF**2
>          NLL=TT/SB
>          NL=(CL-CS/NLL)/CM
>          ALFA=AK1*AK2/SB
>          TANG=CS/VOL
>          RB=ALFA*TANG/NLL
>          PB=-DLOG10(RB)
>    100   WRITE(6,7) VOLUME,PH,CS,NLL,ALFA,RB,NL,PB
>          WRITE(6,13)
>          WRITE(6,14) CNAOH,CM,CLH,CL,VOLI,PK1,PK2
>          STOP
>    110   CONTINUE
>          END
#END OF FILE
```

Example: Partial program output for binding constants determination between 11 and Co^{++}.

```
   VOL        PH        NL(n̄)       PB(pL)
   .600      2.81      0.1560      5.6238
   .800      3.00      0.4030      5.2951
   .840      3.03      0.4666      5.2399
   .880      3.06      0.5476      5.1977
   .920      3.10      0.5999      5.1355
   .960      3.14      0.6696      5.0821
  1.000      3.18      0.7355      5.0272
  1.200      3.37      1.095       4.7635
  1.280      3.46      1.248       4.6563
  1.360      3.55      1.399       4.5405
  1.400      3.61      1.468       4.4713
  1.440      3.68      1.532       4.3927
  1.520      3.83      1.659       4.2167
  1.600      4.08      1.738       3.9438
```

pK_1 and pK_2 were obtained by graphic interpolation in a plot of $\bar{n}$ vs. pL.

$pK_2 = 4.44$ $\qquad$ $pK_1 = 5.22$

APPENDIX III

X-RAY STRUCTURE OF 16b:$ZnBr_2$

(Reproduced from ref. 68 with permission)

The complex 16b:$ZnBr_2$ was obtained by controlled evaporation of equimolar amounts of 16b and $ZnBr_2$ dissolved in DMF, and its structure determined by an X-Ray diffraction method[68].

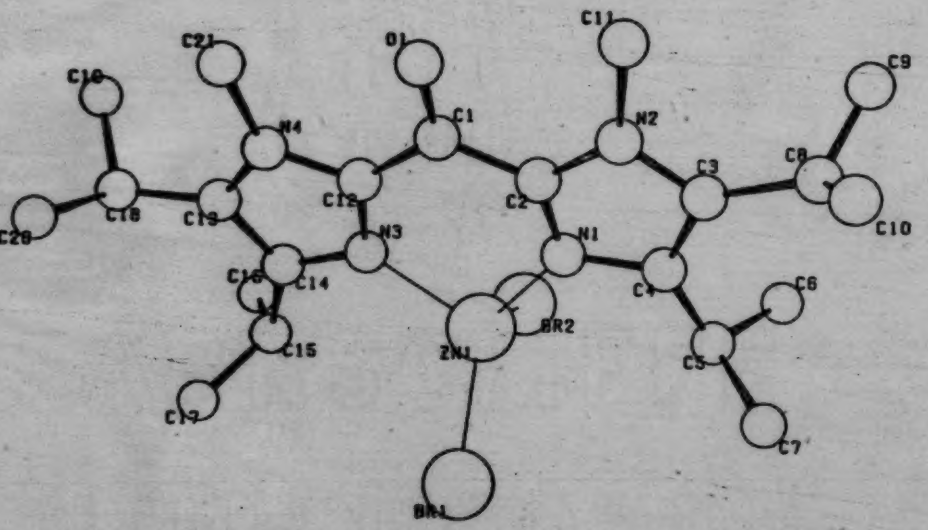

C21
O1
C11
C9
C19
N4
C1
N2
C8
C3
C20
C18
C13
C12
C2
N1
C10
N3
C16
C14
C4
C6
BR2
C15
ZN1
C5
C17
C7
BR1

Fig. AIII.1. PLUTO (Motherwell) drawing of the title compound. Atoms are represented by spheres of arbitrary radius and coordination bonds are represented by single lines.

TABLE AIII.1. Bond lengths (Å) and angles (°)

(e.s.d.'s in parentheses).

C(1)-C(2)	1.47(2)	C(12)-N(4)	1.38(1)
C(1)-C(12)	1.47(2)	C(13)-C(14)	1.39(2)
C(1)-O(1)	1.22(2)	C(13)-C(18)	1.54(2)
C(2)-N(1)	1.31(2)	C(13)-N(4)	1.34(2)
C(2)-N(2)	1.33(2)	C(14)-C(15)	1.50(2)
C(3)-C(4)	1.40(2)	C(14)-N(3)	1.37(1)
C(3)-C(8)	1.50(3)	C(15)-C(16)	1.57(2)
C(3)-N(2)	1.37(2)	C(15)-C(17)	1.51(2)
C(4)-C(5)	1.47(3)	C(18)-C(19)	1.56(3)
C(4)-N(1)	1.41(2)	C(18)-C(20)	1.51(3)
C(5)-C(6)	1.58(3)	C(21)-N(4)	1.47(2)
C(5)-C(7)	1.54(4)	N(1)-Zn	2.041(9)
C(8)-C(9)	1.42(5)	N(3)-Zn	2.053(10)
C(8)-C(10)	1.47(4)	Zn-Br(1)	2.371(2)
C(11)-N(2)	1.56(2)	Zn-Br(2)	2.353(2)
C(12)-N(3)	1.33(1)		

C(2)-C(1)-C(12)	121(1)	C(15)-C(14)-N(3)	122(1)
C(2)-C(1)-O(1)	120(1)	C(14)-C(15)-C(16)	112(1)
C(12)-C(1)-O(1)	120(1)	C(14)-C(15)-C(17)	114(1)
C(1)-C(2)-N(1)	127(1)	C(16)-C(15)-C(17)	111(1)
C(1)-C(2)-N(2)	123(1)	C(13)-C(18)-C(19)	112(1)
N(1)-C(2)-N(2)	110(1)	C(13)-C(18)-C(20)	114(1)
C(4)-C(3)-C(8)	128(2)	C(19)-C(18)-C(20)	113(2)
C(4)-C(3)-N(2)	104(1)	C(2)-N(1)-C(4)	107(1)
C(8)-C(3)-N(2)	128(2)	C(2)-N(1)-Zn	127.3(8)
C(3)-C(4)-C(5)	130(1)	C(4)-N(1)-Zn	125.8(9)
C(3)-C(4)-N(1)	108(1)	C(2)-N(2)-C(3)	111(1)
C(5)-C(4)-N(1)	121(1)	C(2)-N(2)-C(11)	127(1)
C(4)-C(5)-C(6)	114(2)	C(3)-N(2)-C(11)	122(1)
C(4)-C(5)-C(7)	113(2)	C(12)-N(3)-C(14)	108(1)
C(6)-C(5)-C(7)	111(2)	C(12)-N(3)-Zn	125.3(7)
C(3)-C(8)-C(9)	119(2)	C(14)-N(3)-Zn	126.3(7)
C(3)-C(8)-C(10)	115(2)	C(12)-N(4)-C(13)	108.0(9)
C(9)-C(8)-C(10)	108(2)	C(12)-N(4)-C(21)	125(1)
C(1)-C(12)-N(3)	128(1)	C(13)-N(4)-C(21)	127(1)
C(1)-C(12)-N(4)	123(1)	N(1)-Zn-N(3)	91.3(4)
N(3)-C(12)-N(4)	108.8(9)	N(1)-Zn-Br(1)	112.4(3)
C(14)-C(13)-C(18)	126(1)	N(1)-Zn-Br(2)	111.9(3)
C(14)-C(13)-N(4)	107(1)	N(3)-Zn-Br(1)	110.1(3)
C(18)-C(13)-N(4)	126(1)	N(3)-Zn-Br(2)	112.0(3)
C(13)-C(14)-C(15)	131(1)	Br(1)-Zn-Br(2)	116.5(1)
C(14)-C(13)-N(4)	107(1)		

APPENDIX IV

X-RAY STRUCTURE OF 29c:$ZnCl_2$

(Reproduced from Ref. 76 with pemission)

The complex 29c:$ZnCl_2$ was obtained by controlled evaporation of equimolar amounts of 29c and $ZnCl_2$ dissolved in EtOH:DMF, and its structure determined by X-Ray diffraction method.

Fig. AIV.1 shows the structure of the complex. The zinc ion is coordinated equivalently to the three imidazoles as predicted from nmr experiments. The fourth ligand is a chloride ion. An interesting observation is the way the isopropyl groups in the 4-imidazole positions are oriented, allowing only one ligand to enter the cavity and bind the zinc ion. Another interesting feature of the complex is the hydrogen bonding between to imidazole N-H and the carbonyl of two DMF molecules which crystallized with the complex. The X-ray structure of the enzyme suggests that two ligand histidine N-H groups are hydrogen bonded to carbonyl groups[5b]: His 94 to Gln 92, and His 96 to the peptide carbonyl of Asn 244 (Fig. 2).

Some bond lengths and angles are listed in TABLE AIV.1. The P-C bond lengths and the N-Zn distances agree with the ones predicted in page 76. The bond angles around the phosphorus are slightly smaller than predicted.

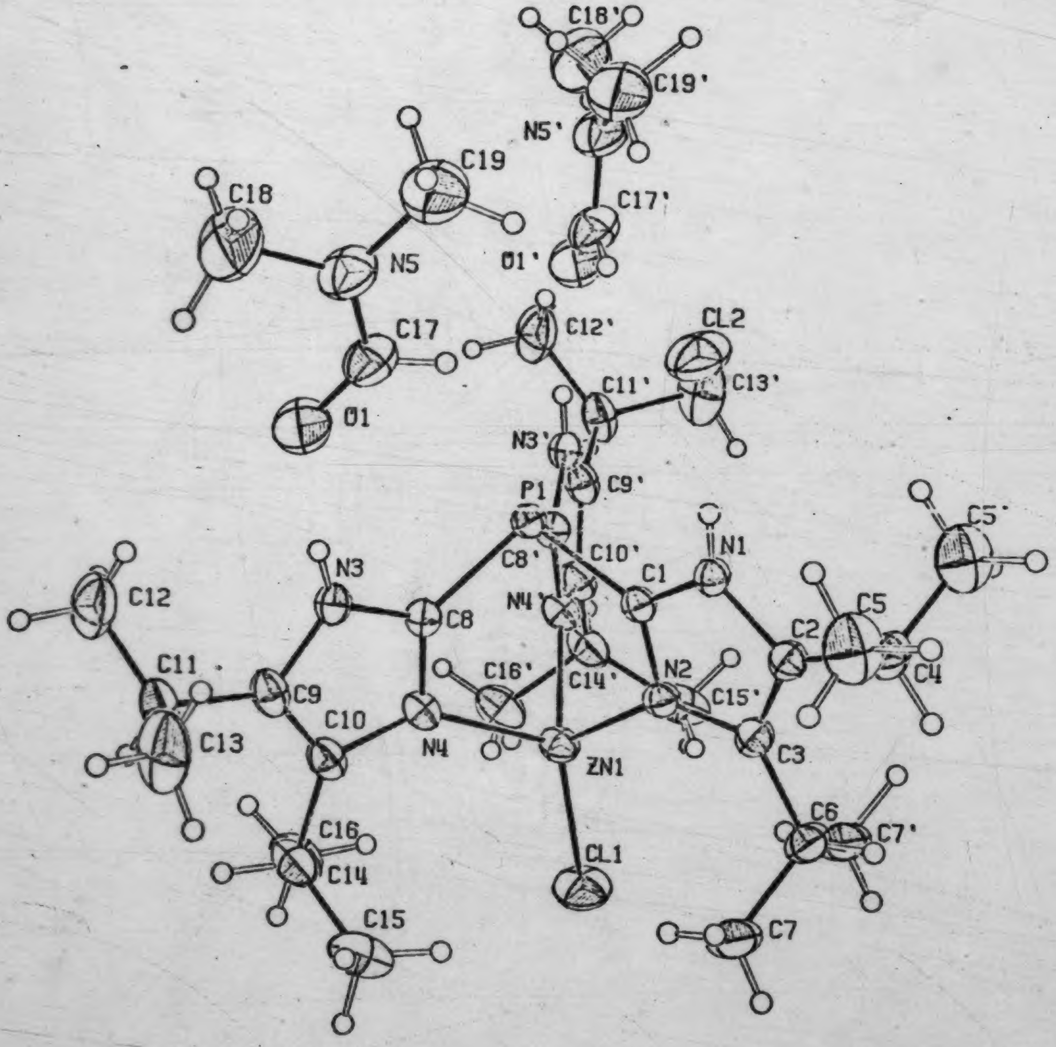

C18'
C19'
N5'
C19
C18
C17'
N5
01'
C17
C12'
CL2
C11'
C13'
01
N3'
C9'
P1
C5'
N1
C8'
C10'
C1
N3
C12
C8
N4'
C5
C2
C11
C9
C16'
C14'
N2
C15'
C4
C10
N4
C13
ZN1
C3
C6
C7'
C16
C14
CL1
C15
C7

Fig. AIV.1. ORTEP drawing of the complex showing two molecules of DMF which crystallized with it. Atoms are represented by 35% probability thermal ellipsoids. Hydrogens are drawn as spheres of arbitrary radium 0.1 Å.

Table AIV.1. Bond lengths (Å) and angles (°)

P(1)-C(1)	1.82	C(8)-N(4)	1.34
P(1)-C(8)	1.82	C(9)-C(10)	1.38
C(1)-N(1)	1.34	C(9)-N(3)	1.37
C(1)-N(2)	1.34	C(10)-N(4)	1.39
C(2)-C(3)	1.38	C(9)-C(11)	1.50
C(2)-C(4)	1.50	C(10)-C(14)	1.50
C(2)-N(1)	1.38	N(4)-Zn	2.06
C(3)-C(6)	1.50	N(2)-Zn	2.06
C(3)-N(2)	1.39	Zn-Cl	2.17
C(8)-N(3)	1.35		

C(1)-P(1)-C(8)	97	C(8)-N(4)-Zn	113
C(8)-P(1)-C(8)^{1}	99	N(2)-Zn-N(4)	96
N(1)-C(1)-N(2)	111	N(4)-Zn-N(4)^{1}	94
N(3)-C(8)-N(4)	110	Cl-Zn-N(2)	120
C(1)-N(2)-Zn	114	Cl-Zn-N(4)	122